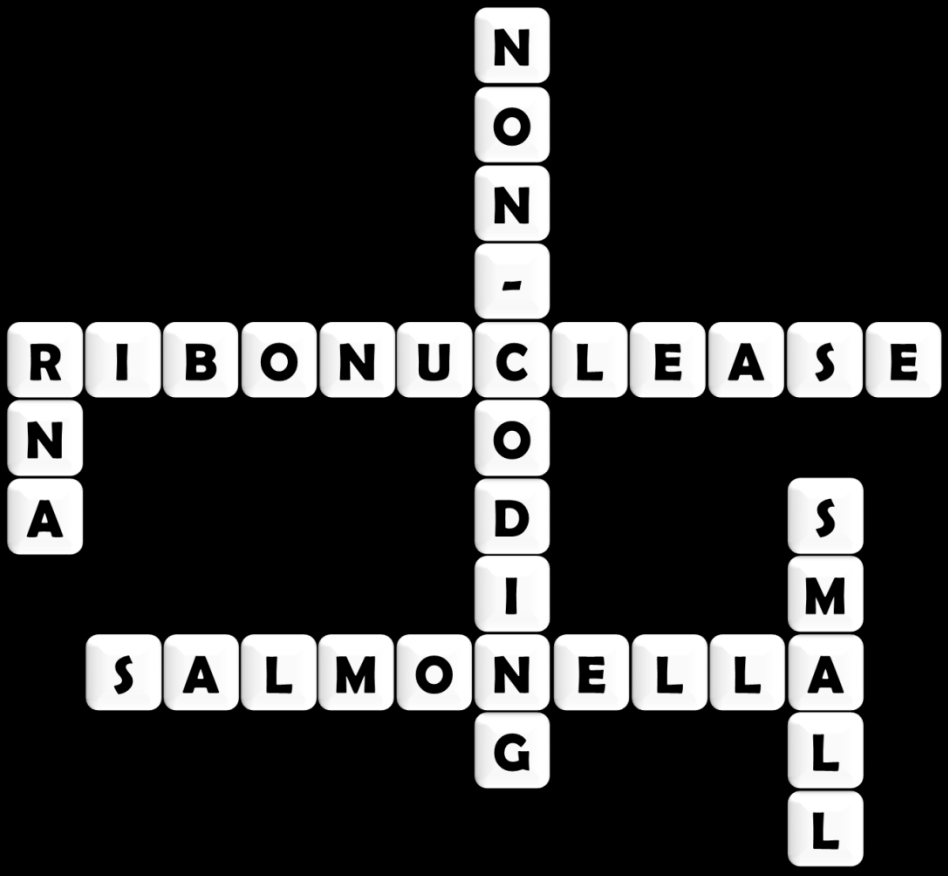
The Role of Small RNAs and Ribonucleases in the Control of Gene Expression in *Salmonella* Typhimurium

Inês de Jesus de Almeida e Silva



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
December,
2012

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Knowledge Creation

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To my family, to Dad, Mum, Eduardo and Pedro

"Every experiment proves something. If it doesn't prove what you wanted it to prove, it proves something else."

-Anonymous

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Table of contents

Abstract

RNAs are important effectors in the process of gene expression. In bacteria, the levels of the transcripts have to be rapidly adjusted in response to constantly changing environmental demands. The cellular concentration of a given RNA is the result of the balance between its synthesis and degradation. RNA degradation is a complex process encompassing multiple pathways. Ribonucleases are the enzymes that directly process and degrade RNA transcripts, regulating their cellular amounts. The rate at which RNA decay occurs depends on the availability of ribonucleases and their specificities according to the sequence and/or the structural elements of the RNA molecule. Several other factors modulate RNA degradation, namely polyadenylation, which plays a multifunctional role in RNA metabolism. Additionally, small non-coding RNAs are crucial regulators of gene expression, and can directly modulate the stability of their mRNA targets. In many cases this regulation is dependent on Hfq, an RNA binding protein which can act in concert with polyadenylation enzymes and is often necessary for the activity of the sRNAs.

The first objective of this Doctoral work was to study the importance of the endoribonucleases RNase III and RNase E on the regulation of the sRNA MicA in *Salmonella* Typhimurium. MicA is a *trans*-encoded sRNA, which down-regulates porin expression in stationary-phase. Its main targets in *Salmonella* are the outer membrane protein OmpA and the LamB maltoporin. In a previous study, we have analyzed the expression profile of MicA under different growth conditions and used distinct *Salmonella* RNase mutants to evaluate the contribution of each of these enzymes in the turnover of this sRNA. In order to further analyse the role of the two main *Salmonella* endoribonucleases, RNase III and RNase E, in the degradation of MicA, both enzymes were overexpressed and purified. The parallel analysis of the *in vivo* sRNA decay in the absence of each of

the two RNases and the *in vitro* activity of both enzymes using MicA as a substrate, revealed that both endoribonucleases are involved in the control of MicA sRNA levels. However, MicA was shown to be cleaved by RNase III when it is in complex with its targets, while RNase E was the responsible for the control of free-MicA levels in the cell. This study allowed us to propose a model for MicA degradation in which RNase III and RNase E perform a different role in the decay of this sRNA.

In the second part of this Dissertation the aim was to examine the role of SraL sRNA in *S.* Typhimurium. SraL was originally identified in *E. coli*, and was afterwards detected in *Salmonella*, particularly in late stationary phase of growth. SraL has been little studied and its biological function was not known at the beginning of this study. Several approaches are currently available to determine the biological role of the sRNAs. In the present work, a proteomic analysis of *Salmonella* strains expressing different SraL levels in the cell enabled the detection of several SraL putative targets. The majority of the changes were detected in proteins involved in several pathways of carbohydrates metabolism, especially those involving glucose. Moreover, the study of the influence of glucose in SraL levels revealed a dependence of the expression of this sRNA on the concentration of this sugar in the growth medium.

In the proteomic analysis, one of the proteins most affected by the different levels of SraL in the cell was the chaperone Trigger Factor. This putative biological function of SraL was investigated further in the third part of this Doctoral work. Trigger factor is the first chaperone encountered co-translationally by most of the nascent amino-acid chains and cooperates with DnaK and GroEL in the folding of newly synthesized proteins. In this study, it was shown that this regulation occurs at the level of the mRNA. The region of interaction between the

sRNA and the mRNA target was predicted by using several bioinformatic tools. Mutagenesis experiments have shown that SraL interacts with the 5'-UTR of *tig* mRNA few nucleotides upstream of its Shine Dalgarno sequence.

The main ribonucleases involved in the post-transcriptional regulation of SraL were previously identified. In the present work, several experiments were performed to study how this sRNA is regulated at the transcriptional level. The results presented in this Dissertation identified the central regulator of general stress response RpoS (σ^S) as a transcriptional regulator of this sRNA. By using Chromatin immunoprecipitation assays, RpoS was shown to bind directly to the SraL promoter region.

The work developed during this Dissertation provided important results that contributed for a better understanding of the biological role of two highly conserved sRNAs, MicA and SraL sRNAs, and their pathways of regulation in the human pathogen *Salmonella* Typhimurium.

Resumo

Os RNAs constituem importantes moléculas efetoras no processo de expressão génica. Em bactérias, os níveis dos transcritos têm que ser rapidamente ajustados em resposta às constantes mudanças no meio ambiente. A concentração celular de um dado RNA é determinada pelo balanço entre a sua síntese e a sua degradação. A degradação do RNA é um processo bastante complexo e que envolve numerosos passos. As ribonucleases (RNases) são as enzimas responsáveis pela regulação da quantidade dos transcritos de RNA, uma vez que atuam no seu processamento e na sua degradação. A taxa de degradação do RNA depende da disponibilidade destas enzimas e também das suas especificidades, de acordo com a sequência e/ou elementos estruturais das moléculas de RNA. Vários outros fatores modulam igualmente a degradação do RNA. A poliadenilação desempenha variadas funções durante o metabolismo do RNA. Adicionalmente, os pequenos RNAs não-codificantes são também reguladores essenciais da expressão génica, visto que modulam diretamente a estabilidade dos seus RNAs mensageiros (mRNA) alvos. Na maioria dos casos documentados, esta regulação é dependente do *chaperone* Hfq. Esta proteína tem a capacidade de se ligar ao RNA e pode eventualmente atuar em conjunto com as enzimas de poliadenilação. Para além disso, o Hfq é normalmente necessário para a própria atividade dos pequenos RNAs.

O primeiro objectivo do trabalho desenvolvido durante esta Dissertação centrou-se no estudo da importância das endoribonucleases RNase III e RNase E na degradação de um pequeno RNA, denominado MicA, em *Salmonella* Typhimurium. Este pequeno RNA regula negativamente a expressão de porinas em fase estacionária. Até à data existem dois alvos descritos para este pequeno RNA em *Salmonella*: a proteína de membrana externa OmpA; e a maltoporina LamB. Num estudo realizado anteriormente, analisámos a expressão do MicA em

diferentes condições de crescimento e usando mutantes de diversas RNases de *Salmonella* de modo a avaliar a contribuição de cada uma destas enzimas no decaímento deste pequeno RNA. Com vista a analisar com maior detalhe o papel das duas principais endoribonucleases de *Salmonella* na degradação deste pequeno RNA, sobre-expressámos e purificámos ambas as enzimas de *Salmonella*. A análise paralela dos resultados obtidos no estudo da degradação deste pequeno RNA *in vivo* na ausência de ambas as RNases e dos resultados obtidos no estudo da atividade *in vitro* de ambas as enzimas revelou que ambas as endoribonucleases estão envolvidas no controlo dos níveis de expressão do MicA. Os estudos efetuados revelaram que o MicA é degradado pela RNase III quando forma complexos com os seus RNAs mensageiros alvos. Adicionalmente, a RNase E é responsável pelo controlo dos níveis do pequeno RNA livre na célula. Os resultados obtidos permitiram-nos propor um modelo de degradação do MicA, no qual a RNase III e a RNase E desempenham funções diferentes no decaímento deste pequeno RNA.

Na segunda parte da presente Dissertação o principal objetivo foi estudar e compreender a função de outro pequeno RNA de *Salmonella*, denominado SraL. Este pequeno RNA foi originalmente identificado em *Escherichia coli*. Mais tarde foi também detetado em *Salmonella*, maioritariamente na fase estacionária tardia de crescimento. Tinham sido já efetuados alguns estudos tendo como base este pequeno RNA. Contudo, aquando do ínicio deste estudo, a sua função biológica na célula não tinha sido ainda identificada. Presentemente conhecem-se vários métodos que permitem a determinação da função biológica dos pequenos RNAs. Neste estudo, foi afetuada uma análise proteómica de várias estirpes de *Salmonella* a expressar diferentes níveis de SraL na célula. Esta abordagem permitiu a deteção de vários alvos putativos deste pequeno RNA. Grande parte das proteínas afetadas pelos diferentes níveis de SraL na célula estão envolvidas

em vários passos do metabolismo dos hidratos de carbono, especialmente os que envolvem a glucose. O estudo da influência da glucose nos níveis do SraL foi também realizado e revelou que a expressão deste pequeno RNA é dependente da concentração deste açucar no meio de crescimento.

A análise proteómica revelou também o *Trigger Factor* como sendo outro alvo putativo do pequeno RNA SraL. Na terceira parte deste trabalho foi investigada com maior detalhe esta função biológica do SraL. O *Trigger Factor* é o primeiro *chaperone* que se liga co-traducionalmente à maioria das cadeias de aminoácidos. Esta proteína coopera com outros dois *chaperones*, DnaK e GroEL, no correto enrolamento das cadeias polipeptídicas recém-sintetisadas. Durante este estudo foi demonstrado que esta regulação se dá ao nível do RNA mensageiro. A região de interação entre o pequeno RNA e o mRNA alvo foi determinada usando várias ferramentas bioinformáticas. Experiências de mutagénese mostraram que o SraL interage com a região não traduzida a 5' do mRNA do *tig*, alguns nucleótidos antes da sua sequência Shine-Dalgarno.

Num estudo anteriormente realizado identificámos as principais ribonucleases envolvidas na regulação pós-transcricional do SraL. No presente estudo, foram efetuadas várias abordagens com vista a compreender como é que este pequeno RNA é regulado transcricionalmente. Com base nos resultados apresentados nesta Dissertação, identificámos o regulador central da resposta ao stress RpoS (σ^S) como sendo o regulador transcricional do SraL. Utilizando ensaios de *Chromatin immunoprecipitation* mostrou-se que o RpoS se liga diretamente à região promotora do SraL.

O trabalho desenvolvido durante esta Dissertação apresenta resultados importantes que contribuem para uma melhor compreensão da função biológica de dois pequenos RNAs altamente conservados, denominados MicA e SraL. Para

além disso, alguns dos processos de regulação destes pequenos RNAs no organismo patogénico *Salmonella* Typhimurium foram também estudados.

List of Publications

Silva I.J., Ortega A.D.*, Viegas S.C.*, García-del Portillo F., Arraiano C.M. 2012. An RpoS-dependent sRNA regulates the expression of a chaperone involved in protein folding. **Accepted with modifications in RNA Journal.** *These authors contributed equally to this work

Reis F., Pobre V., **Silva I.J.**, Malecki M., Arraiano C.M. 2012. The RNB Family of Exonucleases – Putting the 'Dis' in Disease. **Accepted in WIREs RNA**

Silva I.J., Saramago M., Dressaire C., Domingues S., Viegas S.C., Arraiano C.M. 2011. Importance and key events of prokaryotic RNA decay: the ultimate fate of an RNA molecule. **WIREs RNA; 2(6):818-36**

Viegas S.C.*, **Silva I.J.***, Saramago M., Domingues S., Arraiano C.M. 2011. Regulation of the small regulatory RNA MicA by ribonuclease III: a target-dependent pathway. **Nucleic Acids Research; 39(7):2918-30 *These authors contributed equally to this work**

Arraiano C.M., Andrade J.M., Domingues S., Guinote I.B., Malecki M., Matos R.G., Moreira R.N., Pobre V., Reis F.P., Saramago M., **Silva I.J.**, Viegas S.C. 2010. The Critical Role of RNA Processing and Degradation in the Control of Gene Expression. **FEMS Microbiology Reviews; 34(5):883-923**

Andrade J.M., Pobre V., **Silva I.J.**, Domingues S., Arraiano C.M. 2009. The role of 3'-5' exonucleases in RNA degradation. **Progress in Molecular Biology and Translational Science Review, 85:187-229**

Viegas S.C., Pfeiffer V., Sittka A., **Silva I.J.**, Vogel J., Arraiano C.M. 2007. Characterization of the role of Ribonucleases in *Salmonella* small RNA decay. **Nucleic Acids Research; 35(22):7651-64**

Dissertation Outline

This Dissertation is divided into five chapters.

Chapter one consists of a general introduction on RNA degradation mechanisms, where are highlighted the main differences between the RNA degradation pathways for the two main Gram-negative and Gram-positive bacterial models, *Escherichia coli* and *Bacillus subtilis*. This chapter encompasses not only the importance of ribonucleases but also of other players such as small non-coding RNAs, the RNA-chaperone Hfq and Poly(A) Polymerase. Part of this section was published in *WIREs RNA* and the author of this thesis is the first author of this manuscript.

Chapter two describes the investigation of the role of the endoribonucleases III and E in the degradation of MicA sRNA, alone and coupled with its targets. From this work resulted a publication in *Nucleic Acids Research* in which the author of this dissertation played a major contribution and was considered first author.

Chapter three focused in the biological function of SraL in *Salmonella* Typhimurium. Proteomic were performed to identify possible targets of this sRNA. The proteomic results that seemed more relevant were validates by RT-PCR and Northern blot analyses. The importance of the regulation of SraL over the putative targets identified is discussed. The results described in this chapter gave the preliminary data necessary for the work reported in chapter four, and has also information that will be instrumental for other publications.

In chapter four one of the biological roles inferred in the previous chapter for SraL in *Salmonella* Typhimurium was also studied in detail and the transcriptional regulator of SraL sRNA was also determined. The work of this chapter was accepted with modifications in *RNA* and the author of this dissertation is the first author of the manuscript.

To finalize, chapter five comprises an integrated discussion of the global results obtained during this Dissertation, including future perspectives.

Abbreviations

A adenine

Amp ampicillin

A. aeolicus *Aquifex aeolicus*

ATP adenosine triphosphate

bp base pair

B. subtillis *Bacillus subtillis*

BSA bovine serum albumin

°C degree Celsius

C cytosine

cAMP-CRP cyclic adenosine monophosphate-cAMP receptor protein

c-di-GMP cyclic diguanylic acid

cDNA complementary DNA

ChIP chromatin immunoprecipitation

Cm chloramphenicol

cpm counts per minute

CRISPR clustered regularly interspaced short palindromic repeats

CS cold-shock

CSD cold-shock domain

Δ deletion

DTT dithiothreitol

DNA deoxyribonucleic acid

DNase deoxyribonuclease

dsRBD double-stranded RNA binding domain

dsRNA double stranded RNA

E. coli *Escherichia coli*

EDTA ethylenediaminetetraacetic acid

G guanine

g relative centrifugal force

GMP deoxyguanosine monophosphate

h hour

Hfq-coIP-seq Hfq coimmunoprecipitation coupled with RNA-seq

His histidine

HS heat-shock

IgG immunoglobulin G

IP immunoprecipitate

IPTG isoPropyl-β-D-thiogalactopyranoside

kDa kilodalton

L. lactis *Lactococcus lactis*

L. monocytogenes *Listeria monocytogenes*

LB luria-bertani broth

Log logarithm

LPS lipopolysaccharide

M molar/ molarity (mol/L)

M9 minimal medium

Mg magnesium

mg milligram

µg microgram

µl microliter

Mg magnesium

ml milliliter

min minute

mM milliMolar

MOPS 3-(N-morpholino)propanesulfonic acid

mRNA messenger RNA

ng nanogram

nM nanomolar

nt nucleotide

OD optical density

Oligo oligonucleotide

OMP outer membrane protein

^{32}P phosphorus 32 radionucleotide

p p-value

PAA polyacrylamide

PAGE polyacrylamide gel electrophoresis

PAP I poly(A) polymerase I

PBS phosphate-buffered saline buffer

PCN phosphate-carbon-nitrogen

PCR polymerase chain reaction

PDB protein data bank

PMSF phenylmethylsulfonyl fluoride

PNPase polynucleotide phosphorylase

Poly(A) polyadenylate

PPIase peptidyl-prolyl *cis*/*trans* isomerase

psi pressure unit

RBS ribosome binding site

RNA ribonucleic acid

RNAi RNA interference

RNase ribonuclease

r.p.m. rotations per minute

rRNA ribosomal RNA

RT-PCR reverse transcriptase polymerase chain reaction

SD Shine-Dalgarno

SD standard deviation

SDS sodium dodecyl sulphate

siRNA small interfering RNA

SOC super optimal broth with catabolite repression medium

SPI *Salmonella* pathogenicity island

Sr streptomycin

sRNA small RNA

SSC sodium chloride/sodium citrate

S. aureus *Staphylococcus aureus*

T thymine

TAE tris/acetic acid/EDTA

TBE tris/borate/EDTA

TCA tricarboxylic acid cycle

TCS two-component system

TF trigger factor

tmRNA transfer messenger RNA

Tris trishydroxymethylaminomethane (2-Amino-2-(hydroxymethyl)propane-1,3-diol**)**

tRNA transfer RNA

T. thermophilus *Thermus thermophilus*

U uracil

U units

UTP uracil triphosphate

UTR untranslated region

UV ultraviolet radiation

V volt

v/v volume/volume

wt wild-type

w/v weight/volume

Zn zinc

Chapter 1

INTRODUCTION

Part of this chapter was based on:

Silva I.J., Saramago M., Dressaire C., Domingues S., Viegas S.C., Arraiano C.M. 2011. Importance and key events of prokaryotic RNA decay: the ultimate fate of an RNA molecule. **WIREs RNA**; 2(6):818-36

INTRODUCTION

Bacteria have evolved complex regulatory networks in order to adjust their physiology and survive in face of constantly changing environmental conditions. Maintenance of cellular functions relies on the proper expression of genetic information, in which the RNA molecules play a key role. Among RNAs, mRNAs constitute the molecular link between genes and proteins. Because the cellular concentration of a given transcript depends on the rates of its synthesis and degradation, both transcription and degradation control the levels of each protein in the cell. Regulating gene expression at the messenger level is of utmost importance for guaranteeing versatility in the context of the small genome size found in prokaryotes where transcription and translation are coupled. Survival and development in challenging growth conditions require a rapid adaptation of gene expression. In this regard, control of transcription and degradation of mRNA requires less energy and proceeds much faster than translation and protein degradation processes (Russell, 2007). The rate of turnover is not related to the length of the gene; the stability of the gene transcripts seems to be regulated by determinants located in specific mRNA segments and the segments that decay more rapidly can be located anywhere in the mRNA. Moreover, distinct processing may confer differential stability upon the fragments of a polycistronic transcript. The complexity of the RNA degradation process was further revealed with the discovery of non-coding RNAs. Previously overlooked, sRNAs are now known to exert significant regulatory effect on gene expression, and have thus attracted increasing interest due to their regulatory functions, as well as their role in bacterial adaptation and virulence (Repoila and Darfeuille, 2009; Shimoni *et al.*, 2007). Furthermore, the regulation of their cellular levels constitutes an upstream control of gene expression.

In the Introduction chapter is presented the current knowledge on prokaryotic RNA degradation mechanisms. Several questions are discussed like: which are the main players involved? What is their mode of action? Are there any differences between bacterial species? After a brief overview of the genome-wide studies of RNA decay, prokaryotic RNA degradation mechanisms are described. The RNA features and the structural details of ribonucleases, the enzymes that process and degrade RNA, are also discussed. Finally, additional factors which have an impact on RNA stability are focused. Together, these key events determine the ultimate fate of an RNA molecule.

RNA DECAY: GLOBAL APPROACHES

The development of transcriptome analysis has enabled the monitoring of intracellular RNA levels at the genomic scale. The rate of degradation of a given RNA can be estimated by determining its half-life in the cell. Combining inhibition of transcription with microarray technology has proven to be a powerful tool for assessing genome-wide mRNA decay. Most of these global studies in microorganisms have been carried out on the model yeast *Saccharomyces cerevisiae* (García-Martínez *et al.*, 2004; Molin *et al.*, 2009; Wang *et al.*, 2002). mRNA half-lives ranging from 3 to more than 100 min have been observed for this eukaryotic microorganism (Wang *et al.*, 2002), with a majority of transcripts displaying half-lives in the range of 10–20 min (Grigull *et al.*, 2004). Although the available literature is less abundant for prokaryotes, shorter half-lives, with mean values of less than 10 min, have been reported for the bacterial models *Escherichia coli* (Bernstein *et al.*, 2002; Selinger *et al.*, 2003) and *Bacillus subtilis* (Hambraeus *et al.*, 2003). Slightly lower values have been obtained during the exponential growth of *Lactococcus lactis* (Redon *et al.*, 2005) and *Staphylococcus*

aureus (Anderson *et al.*, 2006). The short half-life of bacterial mRNAs is a key factor to allow the rapid adaptation to changing environmental conditions.

Altogether these global studies not only report average values in the same range of magnitude but also highlight a wide variation of half-lives among mRNAs. Furthermore, global stabilities have been observed to be dependent on growth conditions (Anderson *et al.*, 2006; Redon *et al.*, 2005). For example, the average half-life for mRNAs in *L. lactis* increases from 5.8 to 19.4 min when comparing exponential and carbon starvation phases, respectively (Redon *et al.*, 2005). It has also been noted that genes which share related biological functions usually display similar messenger decay rates (Bernstein *et al.*, 2002; Hambraeus *et al.*, 2003; Selinger *et al.*, 2003). For instance, most house-keeping genes have long half-lives. Proteins which are central in *E. coli* protein–protein interaction network also tend to be encoded by stable transcripts (Janga and Babu, 2009).

The majority of RNA molecules are subjected to regulation and, as is the case for mRNA, their decay can be influenced by growth conditions. Independently of the conditions, the two RNA categories involved in protein synthesis, that is, ribosomal and transfer RNAs, are considered to be more stable than mRNA. Although sRNAs were initially believed to be rather stable RNAs, it has since been shown that they can also be quite susceptible to degradation (Massé *et al.*, 2003a; Viegas and Arraiano, 2008). Plasmid-encoded antisense RNAs have a wide range of half-lives, spanning from less than 2 min to more than 32 min (Vogel *et al.*, 2003). While studies undertaken to date have mainly focused on the identification of new sRNAs, tools for assessing sRNA differential expression and stability are becoming increasingly available (Hutzinger *et al.*, 2010) and this will greatly impact the current knowledge of gene expression.

BACTERIAL RNA DEGRADATION MECHANISMS

The original model and the initiator RNase E

Turnover of RNA molecules involves cleavage reactions that are carried out by RNases, a diverse collection of cellular enzymes, whose functions and properties have been elucidated through the study of mutants (Arraiano *et al.*, 2010; Arraiano *et al.*, 1988). Although *E. coli* possesses a plethora of RNases only a few are devoted to the RNA degradation. The conventional model for RNA decay in this bacterium usually begins with an endonucleolytic cleavage at one or more internal sites on the RNA molecule (Figure 1A). Two endonucleases have been associated with the initial cleavage event: RNase III and RNase E. However, RNase E is believed to be the main endonuclease involved in the RNA turnover in *E. coli* (Arraiano *et al.*, 2010). In fact, a recent report shows that in the absence of RNase E 60% of the annotated coding sequences were either increased or decreased in their steady-state levels (Stead *et al.*, 2011). In contrast, only 12% of the coding sequences were affected by the absence of RNase III (Stead *et al.*, 2011).

RNase E is an essential single-stranded endonuclease that exhibits a preference for A/U-rich regions in close proximity to stem-loops (Mackie, 1992; McDowall *et al.*, 1994). This characteristic is also shown by its paralogue, RNase G. This endonuclease, which has a strong resemblance with the amino-terminal portion of RNase E (McDowall *et al.*, 1993), is also involved in the degradation and processing of RNA (Carpousis *et al.*, 2009). Both enzymes display higher activity over substrates bearing a monophosphorylated than over substrates with a triphosphorylated 5′-end (Carpousis *et al.*, 2009). This selectivity results from an RNase E 5′-sensing pocket that binds a monophosphorylated 5′-terminus, while the active site binds and cleaves the RNA internally (Callaghan *et al.*, 2005). Nonetheless, some substrates are cleaved by RNase E regardless of the 5′-

phosphorylation status. This occurs in molecules with multiple single-stranded sites that allow the direct entry of RNase E through a different pathway, called 'bypass' or 'internal entry' (Baker and Mackie, 2003; Kime *et al.*, 2010).

RppH is an RNA pyrophosphohydrolase that removes the pyrophosphate from the 5'-termini and preferentially acts on single-stranded RNA. The discovery of this enzyme presented an alternative pathway in which the initial event is non-nucleolytic (Deana *et al.*, 2008). Conversion of 5'-triphosphate to 5'-monophosphate by RppH provides the ideal substrate for RNase E, and the preference of RppH for single-stranded RNA explains why 5'-stem-loops are mediators of stability. Ribosome loading is also known to mediate RNA stability. A poor ribosome binding site, possibly by increasing the distance between the actively translating ribosomes, exposes putative internal cleavage sites and may increase message instability.

The catalytic domain of RNase E lies in the N-terminal region, which is highly conserved and essential for cell viability (Kaberdin *et al.*, 1998). The C-terminus forms a scaffold for interactions with other proteins, which together form the degradosome, the main RNA degradative complex in *E. coli* (Liou *et al.*, 2001). The RNA degradosome can undergo changes in composition depending on the growth or stress conditions (Gao *et al.*, 2006; Prud'homme-Genereux *et al.*, 2004). For instance, two different RNA helicases are known to associate with RNase E depending on the temperature (Prud'homme-Genereux *et al.*, 2004; Py *et al.*, 1996). This remodelling of the degradosome strongly affects its RNA target spectrum (Gao *et al.*, 2006). In *E. coli* under normal growth conditions, the major components of the degradosome, in addition to RNase E, are the exonuclease PNPase, the helicase RhlB and the glycolytic enzyme enolase (Carpousis *et al.*, 1994; Py *et al.*, 1996). *Pseudomonas syringae*, on the other hand, has selected RNase R as a degradosome component, despite possessing PNPase (Purusharth *et al.*,

2005). This complex of enzymes assures the coordination of the endo- and exonucleolytic degradation of an RNA molecule. After the initial endonucleolytic cleavage step the upstream fragment, lacking the 3'-terminal hairpin, can be readily digested by 3'-exonucleases. The activity of these enzymes is impaired by a 3'-stem-loop, which protects the majority of the primary transcripts (Andrade *et al.*, 2009). The downstream fragment generated after the initial endonucleolytic cleavage is usually more prone to degradation. It bears a monophosphorylated 5'-end and therefore may be the ideal substrate for an additional cleavage by RNase E. The turnover of *malEF* transcript illustrates how the endo- and exonucleolytic enzymes can act in a concerted way. PNPase degradation of *malEF* is only accomplished in the presence of RNase E and RhlB, indicating that the degradosome participates in its degradation (Stickney *et al.*, 2005).

Three exonucleases are mainly involved in RNA decay in *E. coli*: PNPase, RNase R and RNase II (Figure 1A). All of these enzymes degrade RNA processively and non-specifically from the 3'-end. While PNPase is a phosphorolytic exonuclease yielding nucleoside diphosphates as reaction products, both RNase R and RNase II catalyse the hydrolysis of the RNA substrates, producing nucleoside monophosphates. Among the three, only RNase R is able to digest structured RNA by itself (Andrade *et al.*, 2009). The degrading activity of PNPase or RNase II is stalled by the presence of secondary structures (Spickler and Mackie, 2000). However, PNPase can also proceed through extensive folded RNA when acting in association with other proteins. Its association with the helicase RhlB or integration into the degradosome allows the unwinding of the RNA stem-loops (Liou *et al.*, 2002). Surprisingly, the PNPase homologue of *Thermus termophilus*, whose optimal temperature is 65ºC, has been reported to completely degrade RNAs with stable intramolecular secondary structures without the aid of a helicase (Falaleeva *et al.*, 2008). Nonetheless, both PNPase and RNase R require a minimal 3'-overhang of 7–10 unpaired nucleotides

in order to be able to bind and initiate digestion of an RNA molecule (Vincent and Deutscher, 2006). By providing a single-stranded platform for the initiation of the exonucleolytic attack, the degradation of RNA molecules containing 3′-stem-loops is stimulated by the addition of poly(A) tails to the 3′-end of the RNA molecules (for details see below the 'Polyadenylation' section). These poly(A) tails constitute the preferred substrate for PNPase and RNase II (Lisitsky and Schuster, 1999; Marujo *et al.*, 2000).

None of the three 3′-exonucleases seems to be indispensable for *E. coli* growth at optimal temperature. However the combined absence of both PNPase and RNase II or PNPase and RNase R is lethal for the cell, indicating some overlapping role between these exonucleases (Cheng and Deutscher, 2003). For instance, both RNase R and PNPase are involved in the degradation of rRNA fragments, whose accumulation was proposed to lead to cell death (Cheng and Deutscher, 2003). A transcriptome analysis revealed that, although RNase II accounts for 90% of exonuclease activity in the cell, PNPase probably plays a greater role in mRNA degradation than previously thought (Deutscher and Reuven, 1991; Mohanty and Kushner, 2003). RNase II is the major exonuclease involved in *E. coli* RNA decay and other enterobacteriaceae but, this enzyme is absent in several other bacterial species, such as *B. subtilis*, *Legionella pneumophila* and *Streptococcus pneumoniae*, in which RNase R is the only hydrolytic 3′–5′ exonuclease (Charpentier *et al.*, 2008; Domingues *et al.*, 2009). In *B. subtilis* the RNA decay is primarily phosphorolytic and this major activity is attributed to PNPase (Arraiano *et al.*, 2010). Regarding the main exonucleases, PNPase is the only one found in *Streptomyces*, thus constituting an essential protein in these organisms (Bralley *et al.*, 2006). Conversely, the unique exonuclease in *Mycoplasma genitalium* is RNase R, which is thus essential (Hutchison *et al.*, 1999).

The degradative action of the ribonucleases described above releases RNA fragments of 2–5 nucleotides, whose accumulation may be deleterious to the cell (Ghosh and Deutscher, 1999). *E. coli* possesses another exonuclease, termed oligoribonuclease, which acts as a scavenger of these short oligoribonucleotides (Niyogi and Datta, 1975) (see Figure 1A). This essential enzyme processively hydrolyses RNA in the 3′–5′ direction. Overall, oligoribonuclease is a finishing enzyme in RNA metabolism, and the presence of proteins with analogous functions seems to be widespread. Two homologues, NrnA and NrnB, have been described in *B. subtilis* (Fang *et al.*, 2009).

The case of the double-stranded specific RNase III

Even though RNase E has been considered the main enzyme in *E. coli* that catalyses the initial cleavage event, the RNase III family of enzymes has emerged as an important group of endonucleases in the control of RNA stability (Jaskiewicz and Filipowicz, 2008). RNase III deletion in *E. coli* causes a slow growth phenotype (Babitzke *et al.*, 1993), while its homologue in *B. subtilis* is essential for viability (Herskovitz and Bechhofer, 2000). A second *B. subtilis* RNase III-like enzyme (called Mini-III) has been described (Redko *et al.*, 2008). Both enzymes seem to act mostly in bacteriophage mRNA and rRNA processing, since no endogenous mRNA targets are known (Bechhofer, 2009). Interestingly, a recent study performed using the human pathogen *S. aureus* revealed the existence of an overlapping sense/antisense RNA processing by the activity of RNase III (Lasa *et al.*, 2012). This process also occurs in other Gram-positive bacteria such as *B. subtilis*, *Listeria monocytogenes* and *Enterococcus faecalis*.

RNase III is specific for double-stranded RNA and its role in RNA turnover has been associated with the removal of protective stem-loop structures that act as degradation barriers (Arraiano *et al.*, 2010) (Figure 1A). Additionally, RNase III has recently been implicated in the decay of sRNA/mRNA complexes,

which constitute an optimal substrate for this enzyme, upon translational silencing (Viegas and Arraiano, 2008; Viegas *et al.*, 2010). This phenomenon closely resembles siRNA–direct RNA cleavage in eukaryotes, a process that involves enzymes of the RNase III family.

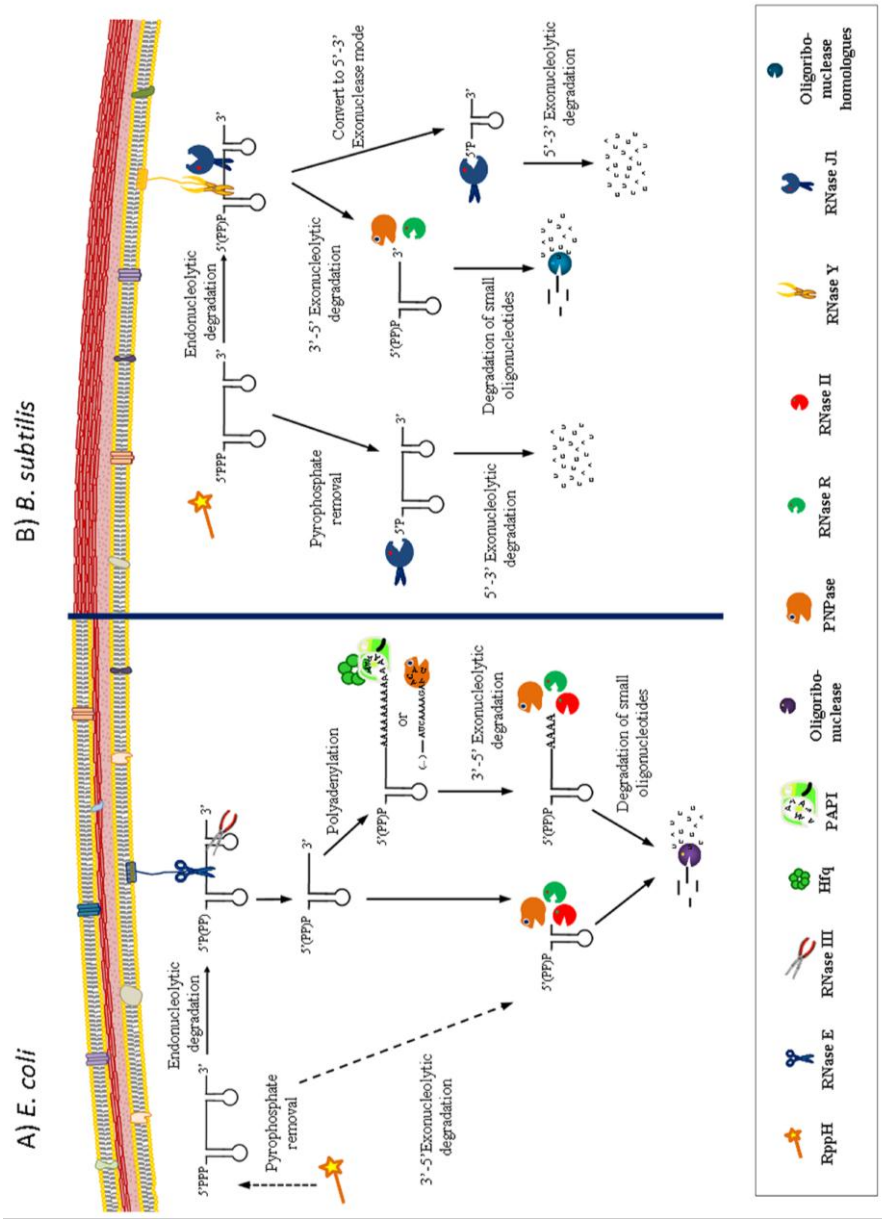

FIGURE 1 - Mechanisms of RNA decay in the *Gram*-negative and *Gram*-positive bacterial models. (A) In *E. coli* the decay of the majority of transcripts starts with an endonucleolytic cleavage by RNase E. The enzyme has a preference for 5′-monophosphorylated substrates. A possible pathway for RNase E cleavage involves a primary cleavage by the RNA pyrophosphohydrolase RppH, which converts the 5′-triphosphorylated terminus of primary transcripts to monophosphate. However, some substrates are cleaved by RNase E regardless of the 5′-phosphorylation status, through an alternative pathway called 'bypass' or 'internal entry', which involves the direct entry of RNase E at single-stranded sites. RNase III is double-stranded specific and can also initiate the decay of structured RNAs. After endonucleolytic cleavage, breakdown products are ready for exonucleolytic digestion by any of the three main exonucleases in this bacterium. Unlike RNase R, both RNase II and PNPase are sensitive to secondary structures. Exonucleolytic activity is promoted by the 3′-polyadenylation of substrates. The activity of PAP I, the main polyadenylating enzyme in *E. coli*, is modulated by the RNA-chaperone Hfq. PNPase can synthesize heteropolymeric tails that also facilitate degradation. Cycles of polyadenylation and exonucleolytic degradation have been proposed as one way to overcome secondary structures. A minor alternative pathway in the cell is the direct exonucleolytic degradation of full length transcripts (represented by a dashed arrow). Exonucleolytic degradation releases short fragments which are subsequently degraded to mononucleotides by oligoribonuclease. **(B)** In *B. subtilis*, transcripts can be degraded from the 5′-end through the 5′–3′ exonuclease activity of RNase J1, or they can be first endonucleolytically cleaved. Since the 5′–3′ exonuclease activity of RNase J1 is blocked by 5′-PPP, before this cleavage the recently discovered BsRppH removes the pyrophosphate. The endonucleolytic cleavage can be either performed by RNase J1/RNase J2 or RNase Y. The breakdown products can be then further degraded by the 3′–5′ exonucleases, PNPase and RNase R (unprotected 3′-ends), or by the 5′–3′ exonuclease activity of RNase J1 (newly generated monophosphorylated 5′-ends). RNase J1 is able to fully degrade its RNA substrates to mononucleotides. The final products released by RNase R and PNPase are further degraded by the oligoribonuclease homologues in *B. subtilis*. Here we represent ribonucleases acting independently. However some of these enzymes can act together in degradation complexes. For instance in *E. coli* the degradosome (RNase E, PNPase, RhlB and enolase) and in *B. subtilis* the putative complex formed by RNase J1/J2, RNase Y, PNPase, the RNA helicase CshA and two glycolytic enzymes.

An alternative mechanism involving different endonucleases

Despite its essential role in *E. coli* RNA turnover, RNase E homologues are absent in numerous bacterial species. This is the case of the model organism *B. subtilis* and is a common characteristic of the low G/C content *Gram*-positive bacteria. For quite some time, a good candidate that could have the analogous role of RNase E was not found, leaving no clue for the process of *B. subtilis* RNA

degradation. One answer came from the discovery of two different ribonucleases, the paralogous RNase J1 and J2, which are present in almost all bacteria lacking RNase E (Even *et al.*, 2005). Curiously, both RNase E and RNase J1 orthologs have been found in *Sinorhizobium meliloti* (Madhugiri and Evguenieva-Hackenberg, 2009). Although there is no sequence homology, the RNase J enzymes share a similar architecture with RNase E (de la Sierra-Gallay *et al.*, 2008) and exhibit equivalent endonucleolytic activity. RNase J1, which is essential for cell viability, is involved in RNA turnover (Mäder *et al.*, 2008) (Figure 1B). Surprisingly, this enzyme is also able to catalyse the exonucleolytic degradation of RNA in the 5'-3' direction (Mathy *et al.*, 2007). To date this is the only 5'-exonuclease known in prokaryotes and its discovery has had important implications in the RNA decay model. The exonucleolytic decay from the 5'-end may explain the stabilizing effect conferred by 5'-stem-loops, 5'-protein binding, 5'-ribosome stalling and the presence of a 5'-triphosphate in *B. subtilis* (Bechhofer, 2009). It has been suggested that this dual-function enzyme (alone or in complex with RNase J2) catalyses not only the endonucleolytic cleavage of an RNA substrate but also continues the degradation of the generated 5'-end by switching to the 5'-exonucleolytic mode (de la Sierra-Gallay *et al.*, 2008). In fact, global analysis of RNase J-depleted *B. subtilis* strains showed an altered abundance for a large number of mRNA transcripts, indicating that this ribonuclease affects gene expression on a global scale (Durand *et al.*, 2012; Mäder *et al.*, 2008). Interestingly, only the exonucleolytic activity of RNase J1 is dependent on the 5'-end phosphorylation status, as it is blocked by triphosphorylated RNA (de la Sierra-Gallay *et al.*, 2008). The complex formed by RNase J1 and J2 changes their individual cleavage activities and specificities (Mathy *et al.*, 2010).

Another insight into the RNA degradation mechanism of *B. subtilis* was the recent discovery of RNase Y, an essential single-stranded endonuclease

(Figure 1B), that shares extensive functional homologies with RNase E. In fact, several reports suggest that the predominant activity of RNase J1 is the 5′-3′ exonucleolytic activity (Condon, 2010; Durand *et al.*, 2012; Lehnik-Habrink *et al.*, 2012; Newman *et al.*, 2011), while RNase Y has an effect on the global mRNA half-life in *B. subtilis* comparable to that of RNase E in *E. coli*. There are several studies reporting an increase of the bulk mRNA half-life in *B. subtilis* upon RNase Y deletion (Durand *et al.*, 2012; Lehnik-Habrink *et al.*, 2011b; Shahbabian *et al.*, 2009). Like RNase E, RNase Y is sensitive to the phosphorylation state of the 5′-end, exhibiting a marked preference for monophosphorylated RNA. Hence, two essential enzymes in *B. subtilis* RNA decay are dependent on a monophosphorylated 5′-end. Indeed, it was recently discovered the existence of a RNA pyrophosphohydrolase in *B. subtilis* (BsRppH) (Richards *et al.*, 2011). BsRppH was shown to remove the γ and β phosphates from the 5′-end of the RNA as orthophosphate (Richards *et al.*, 2011), whereas the one from *E. coli* releases them mainly as a pyrophosphate (Deana *et al.*, 2008). Interestingly, the results obtained with *rppH* deletion mutant suggested the existence of other(s) RNA pyrophosphohydrolase(s) in *B. subtilis* (Richards *et al.*, 2011).

Based on the amino acid sequence, RNase Y seems to comprise at least four domains: a short N-terminal *trans*-membrane domain (Hunt *et al.*, 2006); a coiled-coil domain seemingly important for oligomerization (Lehnik-Habrink *et al.*, 2011a); a KH domain required for RNA binding; and an HD domain that contains the catalytic site (Condon, 2003). The presence of an N-terminal *trans*-membrane domain in RNase Y suggests membrane localization further extending the analogy to RNase E (Shahbabian *et al.*, 2009) (Figure 1B). In fact, the proper membrane localization of RNase Y is essential for *B. subtilis* (Lehnik-Habrink *et al.*, 2011a).

Evidence for the presence of a complex involving RNase Y, RNase J1/J2, PNPase, the RNA helicase CshA and two glycolytic enzymes, enolase and

phosphofructokinase, has been reported (Commichau *et al.*, 2009). This complex brings together some of the degrading activities necessary to achieve full degradation of an RNA molecule. The RNA fragments released by the RNase Y endonucleolytic cleavage could be good substrates for the 3'-exonucleolytic activity of PNPase and for the 5'-exonucleolytic degradation by RNase J1/J2 (Bechhofer, 2009). This putative degradosome-like complex indicates that the presence of such an RNA degradative machine may be a common feature in prokaryotes, even those that lack an RNase E homologue. Nonetheless, the existence of this complex in *B. subtilis* is still controversial. The presence of a degradosome-like complex in this organism has been challenged by the failure of isolating it as a complex in its native state and by the absence of detection of degradosome interactions in yeast two- and three-hybrid screens (Mathy *et al.*, 2010). However, these screens failed to detect also the established self-interactions of the RNase J1 and J2 (Commichau *et al.*, 2009; de la Sierra-Gallay *et al.*, 2008; Newman *et al.*, 2011). Accordingly, the current knowledge does not yet allow accurate conclusions about the presence or absence of an RNA degradosome in this bacterium.

ROLE OF RNA DEGRADATION IN QUALITY CONTROL

Gene mutation, DNA damage, or transcriptional errors may generate damaged mRNAs that are unsuitable for protein synthesis. Additionally, translational frame-shifting can also lead to aberrant proteins, whose accumulation may be detrimental to the cell. Hence, bacteria have evolved efficient quality control mechanisms engaged in the rapid degradation of these abnormal transcripts and proteins.

One of the common errors is the presence of a premature stop codon in some messages, which produces a truncated protein. In *E. coli* the fast degradation of messages carrying a premature stop codon is thought to begin with a 5'-independent RNase E cleavage at internal sites exposed by the premature release of ribosomes (Baker and Mackie, 2003). The resulting RNA molecules are further degraded through the pathways described above.

Messages without an in-frame stop codon may lead to ribosome stalling at the 3'-end, significantly affecting translational efficiency. An elegant surveillance pathway, termed *trans*-translation, targets deficient proteins and mRNA for degradation while rescuing stalled ribosomes (see (Keiler, 2008; Richards *et al.*, 2008) for a review). This process relies on the association of two molecules: a sRNA called transfer-messenger RNA (tmRNA), and a small RNA-binding protein (SmpB), whose homologues have been identified in every sequenced eubacterial genomes. Some situations that delay the progress of ribosomes during translation were also observed to elicit tagging by the *trans*-translation machinery in a process that involves RNase II (Garza-Sánchez *et al.*, 2009; Richards *et al.*, 2008).

The rapid removal of the defective messages that lead to ribosome stalling is of utmost importance in the prevention of future stalling events. RNase R is associated with tmRNA and SmpB and is involved in the tmRNA-mediated decay (Karzai and Sauer, 2001) as well as in the processing of tmRNA (Cairrão *et al.*, 2003). The mechanism that influences the loading of RNase R onto the defective mRNAs was recently highlighted, whereby RNase R was shown to be recruited through interactions mediated by its C-terminal lysine-rich domain in a SmpB-tmRNA-dependent manner (Ge *et al.*, 2010). Indeed, this domain was previously suggested as being involved in interactions with SmpB (Liang and Deutscher, 2010).

Finally, errors in macromolecular processes like tRNA and rRNA synthesis also occur. Li and co-workers had shown that an aberrant precursor tRNA molecule is degraded by a mechanism that involves PAP I and PNPase (Li *et al.*, 2002), leading to the proposition that polyadenylation could serve as a signal to promote degradation of defective tRNAs. PNPase and RNase R have been implicated in the removal of defective rRNAs (Cheng and Deutscher, 2003).

STRUCTURAL DETERMINANTS OF RNA DEGRADING ENZYMES

The intrinsic degradative nature of ribonucleases and the fact that they share the cellular environment with a pool of different types of RNA molecules raises some questions: How do these 'molecular killers' specifically select their targets? What are the structural determinants that allow these enzymes to spare some molecules while dictating the destruction of others? As underlined above there are several features of the RNA molecules that are key factors in determining their fate. The interplay between the structural determinants of the enzymes and their specific degradation preferences, depending on the characteristics of each RNA molecule, seems to dictate the final decision.

RNase E

RNase E, the main endonuclease of numerous species including *E. coli*, organizes its catalytic domain, as a dimer of dimers in a final homotetramer quaternary structure (Callaghan *et al.*, 2005) (Figure 2). Each protomer contains the following structural domains: S1, RNase H, DNase I and a small domain that is responsible for dimer–dimer interaction. The arrangement of the domains within each dimer resembles the blades and handles of an open pair of scissors. The crystal structure explains some features of the enzyme and suggests a

mechanism for RNA recognition and cleavage (Koslover *et al.*, 2008). The influence of 5'-phosphorylation is a consequence of the pocket formed between the S1 and the RNase H subdomains, which binds 5'-monophosphorylated RNA and promotes downstream degradation. After binding, a conformational change induced by the movement of the RNA-binding domains clamps the substrate down and organizes the active site (Koslover *et al.*, 2008). The catalytic site contains conserved residues of the DNase I domain and a single metal-binding site that coordinates an Mg^{2+} ion implicated in catalysis. The internal flexibility within the quaternary structure may be related to the deformation required to accommodate structured RNA for processing by internal entry. An amphipathic segment (called segment A) at the C-terminal region of RNase E directs the enzyme to the inner membrane (Khemici *et al.*, 2008). It was recently published the existence of another RNase E-membrane binding interaction involving the catalytic domain of the enzyme (Murashko *et al.*, 2012). The N-terminal RNase E membrane binding alters its enzymatic activity by increasing the substrate affinity, and affects the secondary structure of the catalytic domain stabilizing the folding state of the protein (Murashko *et al.*, 2012). Membrane localization of this enzyme could be determinant for spatial discrimination of the RNA substrates.

RNase III

E. coli RNase III is the prototype of the RNase III family of enzymes, which includes eukaryotic enzymes such as Dicer and Drosha. The bacterial enzymes are the simplest, containing an N-terminal endonuclease domain (NucD) characterized by a 9-residue consensus sequence known as the RNase III signature motif, and a double-stranded RNA binding domain (dsRBD) in the C-terminus. The enzyme is active in the homodimeric form and the crystal structure of the *Aquifex aeolicus* RNase III endonuclease domain shows that dimerization creates a large valley that accommodates dsRNA (Figure 2). The catalytic centers

are found in the dimer interface, one at each end of the valley. Although dsRNA binding is governed by the combined dsRBD of the dimer, the NucD domains contribute to substrate specificity (Gan *et al.*, 2008). Substrate selection consists of a combination of structural and sequence elements, such as the strength of base pairing, the occurrence of specific nucleotide pairs and the helix length (Pertzev and Nicholson, 2006). Several conserved residues in the catalytic centers were shown to be essential for catalysis, which involves amino acids from both subunits, and thus, dimerization of the NucD domains is necessary for RNase III function (see (Gan *et al.*, 2006) and references therein). The crystal structure indicates that a single RNA cleavage event occurs on each strand of the dsRNA within each cleavage site, generating products with a two-base 3′-overhang. Mg^{2+} is required for the formation of a catalytical competent protein–RNA complex and two divalent cations are coordinated by each active site. When Mg^{2+} is absent the RNA is bound outside the catalytic valley (Gan *et al.*, 2008). Indeed, RNase III is also known to be involved in the control of gene expression by binding a dsRNA molecule without cleaving. In this non-catalytic functional form the enzyme plays the role of a dsRNA-binding protein (Blaszczyk *et al.*, 2004).

RNase J

RNase J is unique among bacterial RNases in that it possesses both endo- and 5′–3′ exonucleolytic activity (de la Sierra-Gallay *et al.*, 2008). The crystal structure of an RNase J homolog of *T. thermophilus* (sharing 43% and 39% sequence identity with *B. subtilis* RNase J1 and J2, respectively) was determined in the closed state, unable to accommodate the bulk of an RNA structure (de la Sierra-Gallay *et al.*, 2008) (Figure 2). More recently, the structures of *T. thermophilus* RNase J in complex with short RNA oligomer and of *B. subtilis* RNase J1 with an open conformation suitable for binding substrate RNA were

reported (Dorléans *et al.*, 2011; Newman *et al.*, 2011). In both structures, while there are small differences in the detail of RNA binding, the general mode of binding and the path taken by the RNA is the same.

The enzyme is active as a dimer in solution, and each monomer contains three distinct domains: a metallo-β-lactamase core, a β-CASP and a C-terminal domain. This last domain may be involved in substrate recognition and maintenance of the dimeric state. Two Zn^{2+} ions, essential for catalysis, are coordinated in an octahedral environment by residues located deep in the cleft between the β-lactamase core and the β-CASP domain. Specific attack of 5′-monophosphorylated transcripts is related to a binding pocket near the catalytic center that precludes the accommodation of a 5′-triphosphorylated substrate. Only one catalytic center was obvious from the structures, suggesting that a single active site is responsible for the dual activity of RNase J. In fact, the model of *B. subtilis* RNase J1 bound to RNA contains a significant region of unoccupied space at the free 5′-end of the RNA perhaps explaining how both endonuclease and 5′-to-3′ exonuclease activities are incorporated in the same active site (Newman *et al.*, 2011). Indeed, the 5′-monophosphate generated after the endonucleolytic cleavage may be directly placed in the binding pocket with a single translocation of the RNA molecule, allowing RNase J to start the exonucleolytic degradation. In both *B. subtilis* and *T. thermophilus* "open" state structures it is evident the existence of two channels that allow the 5′-3′ exonucleolytic activity of RNase J1 (Dorléans *et al.*, 2011; Newman *et al.*, 2011). There is an RNA binding tunnel formed between the β-CASP and metallo-β-lactamase domains into which the 5′-terminus of the substrate enters to access the active site, and a negatively charged RNA exit channel on the other side of the ribonuclease to evacuate the cleaved nucleotides. Consistent with the absence of sequence specificity by RNase J, most of its contacts with the RNA involve sugar-phosphate backbone rather than the bases (Newman *et al.*, 2011).

RNase II

E. coli RNase II is the prototype of the RNase II family of enzymes, a widespread family that also includes RNase R and the catalytic subunit of the eukaryotic exosome, Rrp44/Dis3. The overall crystallographic structure of *E. coli* RNase II reveals a monomeric protein with four domains: three RNA binding domains comprising CSD1 and CSD2 in the N-terminal region, and an S1 fold at the C-terminus; and one RNB catalytic domain in the central region, which is a hallmark of the RNase II family of proteins (Amblar *et al.*, 2006; Frazão *et al.*, 2006; Zuo *et al.*, 2006) (Figure 2). The RNA binding domains are grouped together in one side of the structure, and play a role in the RNA substrate selection and binding (Frazão *et al.*, 2006; Matos *et al.*, 2009).

Interestingly, a truncated enzyme lacking all the RNA binding domains is still able to degrade RNA (Matos *et al.*, 2009; Vincent and Deutscher, 2009b). The structure of the enzyme in complex with an RNA molecule reveals two main non-contiguous interaction points between the protein and the RNA fragment. A 10-nucleotide segment is the shortest RNA able to retain both contacts, explaining why RNase II becomes distributive on substrates shorter than 10 nt (Frazão *et al.*, 2006). A single amino acid change in the catalytic region alters the final end-product from 4 to 10 nt, probably due to loosening of the RNA substrate at the catalytic site (Barbas *et al.*, 2008). The access to the catalytic pocket is restricted to single-stranded RNAs by steric hindrance, explaining the inability of RNase II to degrade dsRNA. A specific interaction with ribose rings precludes DNA cleavage (Frazão *et al.*, 2006). Several residues in the catalytic region are important for catalysis (Amblar and Arraiano, 2005; Barbas *et al.*, 2008; Barbas *et al.*, 2009; Frazão *et al.*, 2006); however, only Asp209, which is involved in the coordination of Mg^{2+}, is essential (Barbas *et al.*, 2008). Interestingly, substitution of one conserved glutamine gives rise to an enzyme with highly increased RNA-binding and

exonucleolytic activity (120-times higher), consequently called 'super-enzyme' (Barbas *et al.*, 2009).

RNase R

RNase R shares a similar domain organization with RNase II, and a three-dimensional model of this enzyme has been proposed based on the structure of RNase II (Barbas *et al.*, 2008). Besides the domains identified in RNase II, RNase R also contains a helix-turn-helix in the N-terminus (Domingues *et al.*, 2009), and a highly basic region after the S1 fold at the C-terminus (Vincent and Deutscher, 2009b). The RNB domain of RNase R alone is sufficient to bind and degrade an RNA duplex (Matos *et al.*, 2009; Vincent and Deutscher, 2009b). Paradoxically, when the RNA-binding domains are present in the wild-type enzyme a short 3'-overhang is necessary in order to initiate degradation. These domains are essential for binding and recruitment of 3'-tailed RNA molecules. The two CSD domains appear to play a role on the recognition of the substrates for degradation, whereas the S1 domain is most likely required to position substrates for efficient catalysis (Matos *et al.*, 2009; Vincent and Deutscher, 2009b). The RNA-binding regions (S1, CSD1, and most importantly CSD2) have been suggested to possess intrinsic helicase activity (Awano *et al.*, 2010), a hypothesis that still needs further experimental support. Mutation analysis in the nuclease domain identified important residues for the nuclease activity and for substrate binding, which may contribute to the ability of RNase R to degrade structured RNAs (Matos *et al.*, 2009; Matos *et al.*, 2011; Vincent and Deutscher, 2009a). Nevertheless, only the resolution of its three-dimensional structure will allow full understanding of its remarkable mode of action.

PNPase

PNPase belongs to the PDX family of exonucleases, which also includes the core of the exosome in archaea and eukaryotes (Pruijn, 2005). The crystal structures of *Streptomyces antibioticus*, *Caulobacter crescentus* and *E. coli* PNPase reveal a homotrimeric subunit organization with a ring-like architecture (Hardwick *et al.*, 2012; Nurmohamed *et al.*, 2009; Shi *et al.*, 2008; Symmons *et al.*, 2000) (Figure 2). This structure closely resembles the doughnut-like shape of the archaeal and the eukaryotic exosomes (Pruijn, 2005). Each PNPase monomer comprises two RNase PH domains (PH1 and PH2) forming the catalytic site (PNPase core). While the C-terminal RNase PH domain catalyses the phosphorolytic attack of RNA, the N-terminal domain contributes to the ring-like quaternary structure of the trimeric PNPase assembly (Symmons *et al.*, 2000). These two domains are connected by an α-helical domain. Several mutations introduced into the PNPase core have been shown to influence phosphorolytic and polymerase activities of the enzyme (Briani *et al.*, 2007; Jarrige *et al.*, 2002). In addition, two RNA-binding domains S1 and KH have been found in the C-terminus. These domains are required not only for proper binding, but also contribute to the formation of a more stable trimeric structure (Amblar *et al.*, 2007; Matus-Ortega *et al.*, 2007; Shi *et al.*, 2008). In the quaternary structure, the RNA-binding domains are grouped in one face of the trimer, while the active site is located in the opposite side. The association of the three subunits encloses a central channel through which the RNA molecule travels in the direction of the active site. A properly constricted channel and the conserved basic residues in the neck region play critical roles in trapping RNA for processive degradation. RNA translocation is dynamic, and PNPase undergoes conformational changes at the central channel and its neighbouring regions, while directing the RNA to the active center (Hardwick *et al.*, 2012; Nurmohamed *et al.*, 2009; Shi *et al.*, 2008;

Symmons *et al.*, 2000). Mg^{2+} is required for PNPase enzymatic activity, though Mn^{2+} can also support catalysis. The metabolite citrate has recently been shown to directly modulate the enzyme's activity, connecting RNA degradative pathways with the central metabolism (Nurmohamed *et al.*, 2011). Moreover, PNPase was recently shown to be directly activated by the second messenger c-di-GMP (Tuckerman *et al.*, 2011).

In this section, it were highlighted the structural features of the ribonucleases that are specifically involved in the recognition of a given RNA molecule. In light of the enzymes' spatial architecture, it was covered how both the sequence and structural elements within the transcript regulate both its rate of decay and the primary nucleases involved. Furthermore, it was shown that some ribonucleases can also be influenced by metabolites and by the spatial compartmentalization within the cell, which may modulate their access to different RNA molecules.

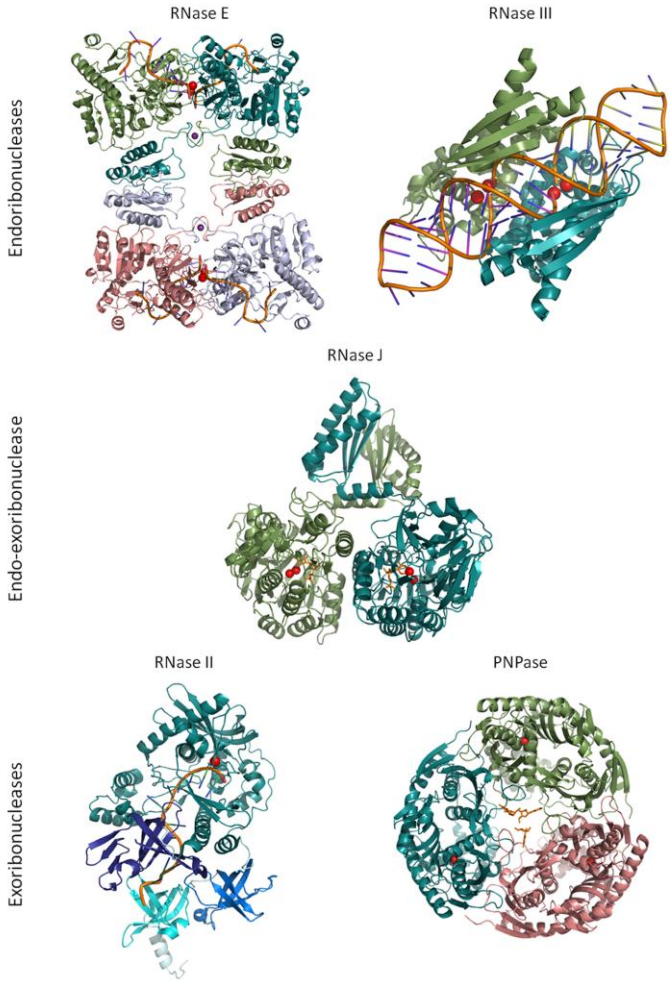

FIGURE 2 - Structures of RNA degrading enzymes in complex with RNA substrates. On the top of the image are shown the crystal structures of endonucleases (catalytic domain of *E. coli* RNase E, PDB ID 2C4R, on the left; *A. aeolicus* RNase III, PDB ID 2NUF, on the right). The crystal structures of exonucleases are on the bottom (*E. coli* RNase II, PBD ID 2IX1, on the left; *E. coli* PNPase, PDB ID 3GCM, on the right). The crystal structure of *T. thermophilus* RNase J (PDB ID 3BK2), which has a dual function as endo- and exonuclease, is in the middle. RNA substrates in complex with the enzymes are coloured in orange and the metal ions that assist catalysis are shown as red spheres. Purple spheres denote the Zn^{2+} ions important for maintenance of the principal dimers in the RNase E quaternary structure. Otherwise the colours are unrelated to the functional domains of the enzymes, but represent different protomers in the quaternary structures except in RNase II, which is active as a monomer. In this case, each colour identifies a different domain (CSD1 and CSD2 are shown in cyan and light blue, respectively; S1 is shown in dark blue and the

catalytic domain RDB is coloured in dark cyan). A model for RNase R has been proposed but the structure is not yet available. However functional and structural data available indicates that the structure will be quite similar to RNase II. Structures were drawn using PyMOL (http://pymol.sourceforge.net).

OTHER PLAYERS

Polyadenylation

Polyadenylation is a post-transcriptional event that involves the addition of untemplated adenosine residues to the 3'-ends of RNA substrates. This widespread phenomenon occurs in bacteria, organelles, archaea, and in the nucleus and cytoplasm of eukaryotic cells. Eukaryotic poly(A) tails, present in the majority of mRNAs, are usually long, uniform in length and have traditionally been viewed as a stabilizing element. However, recent studies have also shown evidence of a polyadenylation-induced decay of non-functional RNAs in eukaryotes (Houseley and Tollervey, 2009). Prokaryotic polyadenylated transcripts are generally low in abundance and poly(A) tails are very short and unstable. This fact, together with a lack of evidence for a physiological role, made polyadenylation in bacteria almost overlooked for several years after its discovery. It has been estimated that less than 2% of total RNA in *E. coli* is polyadenylated. Despite this intriguingly low percentage, a transcriptome comparison between the wild-type and a strain defective in polyadenylation has indicated that the majority of transcripts (~90%) undergo some degree of polyadenylation during exponential growth, either as full-length transcripts or decay intermediates (Mohanty and Kushner, 2006). The rapid turnover of polyadenylated mRNAs accounts in part for their low abundance.

Poly(A) tails provide a single-stranded extension region that works like a 'toe-hold' upon which exonucleases can bind and initiate decay (Dreyfus and Régnier, 2002) (Figure 1A). Polyadenylation therefore facilitates exonucleolytic

activity. Paradoxically, the higher affinity of RNase II for these poly(A) stretches has been shown to protect mRNAs from degradation, as this enzyme degrades the tails that allow PNPase and RNase R to proceed through secondary structures (Marujo *et al.*, 2000; Mohanty and Kushner, 2003).

Initially identified 50 years ago in *E. coli* (August *et al.*, 1962), Poly(A) Polymerase I is responsible for approximately 90% of the polyadenylating activity in the cell (Mohanty and Kushner, 2011). The enzyme catalyzes the addition of homopolymeric poly(A) tails (15–30 nt long in average) to 3'- hydroxyl termini of RNA molecules using ATP as a substrate (Mohanty and Kushner, 2006; Mohanty *et al.*, 2004; O'Hara *et al.*, 1995). However, deletion of its cognate gene (*pcnB*) has only a moderate effect on growth (Cao and Sarkar, 1992) and does not abolish all the polyadenylating activity in the cell (Mohanty and Kushner, 2000). PNPase can also synthesize A-rich heteropolymeric extensions through its reverse activity (synthetic instead of degrading) (Mohanty and Kushner, 2000) although to a much lesser extent. This PNPase activity has recently been shown to change in response to adjustments of cyclic-di-GMP levels in an O_2-dependent way (Tuckerman *et al.*, 2011). In many prokaryotic organisms, archaea, and organelles of prokaryotic origin lacking a PAP I protein, polyadenylation has been shown to be carried out by PNPase (Slomovic and Schuster, 2011). It has been speculated that the evolutionary precursor of PNPase was the first enzyme to produce these tails. Much later, PAP I would have been acquired by bacteria such as *E. coli*. The enzyme, already specific for ATP (the 'energy currency' of the cell), produced the dominant homopolymeric poly(A) tails. *B. subtilis*, in which as much as 15–25% of total RNA was estimated to be polyadenylated (Gopalakrishna *et al.*, 1981), lacks an identifiable PAP I homologue. Nonetheless, similar polyadenylated and heteropolymeric ends have been observed at the 3'-ends of RNA isolated from wild-type and PNPase mutant strains, indicating that PNPase is not the only

enzyme responsible for the addition of nucleotides to the 3'-end of RNAs in this organism (Campos-Guillén *et al.*, 2005).

Regarding the nature of the poly(A) tail, several observations indicate that transcripts which terminate in a Rho-dependent fashion tend to contain only heteropolymeric tails generated by PNPase. On the other hand, Rho-independent transcription terminators serve as polyadenylation signals for PAP I (Mohanty and Kushner, 2006; Mohanty *et al.*, 2004). Moreover, heteropolymeric tails are mainly added to breakdown products, whereas poly(A) tails are added to both breakdown and full-length transcripts (Mohanty and Kushner, 2011). In stationary phase, the addition of heteropolymeric tails is predominant over the homopolymeric tails commonly found in exponentially growing cells (Cao and Sarkar, 1997). The lack of energy resources in stationary phase could be a reason for this choice, since the generation of adenylated tails at the expense of several ATP molecules is more justifiable under exponential growth (Cao and Sarkar, 1997). Moreover, polyadenylation promotes high mRNA turnover rates which are characteristic of the exponential phase (Mohanty and Kushner, 2000).

In both bacteria and organelles, RNA breakdown products generated by endonucleolytic cleavages are considered the most favoured substrates for 3'-tailing (Goodrich and Steege, 1999; Haugel-Nielsen *et al.*, 1996; Lupold *et al.*, 1999; Perrin *et al.*, 2004). Single-stranded segments at either 5'- or 3'-end of RNA molecules and monophosphorylation at an unpaired 5'-terminus were reported to increase the RNA susceptibility to polyadenylation by PAP I (Feng and Cohen, 2000). This suggests that the endonucleolytically-generated RNA fragments containing single-stranded monophosphorylated 5'-termini would be preferential substrates for 3'-end polyadenylation. However, this is not always required as transient poly(A) tails have been found at the native 3'-ends of RNA molecules (Dreyfus and Régnier, 2002). In *E. coli*, the low level of PAP I (32–50 molecules per cell) is also likely to be a limiting factor in substrate selection (Mohanty and

Kushner, 2006; Mohanty *et al.*, 2004). PAP I over-synthesis is highly toxic, which may be the reason for such a low enzyme level (Cao and Sarkar, 1992; Mohanty and Kushner, 1999).

It has long been presumed that 3′-polyadenylation was only restricted to mRNAs, nevertheless PAP I and PNPase can polyadenylate almost any RNA species, including rRNAs, tRNAs, and sRNAs. Interestingly, while the tails found on rRNAs resemble the ones found on mRNAs, the tails on tRNAs and sRNAs tend to be very short (1–8 nt) (Argaman *et al.*, 2001; Xu *et al.*, 1993). A few sRNAs have been reported to be destabilized by polyadenylation (Argaman *et al.*, 2001; Dam Mikkelsen and Gerdes, 1997; Reichenbach *et al.*, 2008; Söderbom *et al.*, 1997; Viegas *et al.*, 2007). Polyadenylation was also implicated in the promotion of the exonucleolytic degradation of defective tRNAs (Li *et al.*, 2002). A defective $tRNA^{TRP}$ does not accumulate to the normal wild-type levels due to the rapid degradation of its precursor. Using PAP I and/or PNPase mutant strains, it has been proposed that polyadenylation of the defective precursor serves as a signal to promote its fast degradation by PNPase (Li *et al.*, 2002).

PAP I has been reported to have physical interactions with Hfq, PNPase, RNase E, and the RNA helicase RhlB (Mohanty *et al.*, 2004; Raynal and Carpousis, 1999), which suggests that the polymerase could act as part of a multiprotein complex (Mohanty *et al.*, 2004). It has been demonstrated in *E. coli* that PAP I is localized either in the membrane or in cytosol, depending on the growth phase (Carabetta *et al.*, 2009; Jasiecki and Wegrzyn, 2005). Such localization may, however, be indirect through a loose association with RNase E (Khemici *et al.*, 2008; Liou *et al.*, 2001). The release of PAP I from the membrane in the transition from the exponential to the stationary phase has been found to be dependent on the adaptor protein SprE (RssB), previously known for its role in governing the stability of the alternate σ factor RpoS (Carabetta *et al.*, 2009). SprE has also been

reported to be required to maintain the association of PAP I and Hfq with the degradosome during stationary phase (Carabetta *et al.*, 2010). In addition, microarray data have revealed that polyadenylation and turnover of specific *E. coli* transcripts can be modulated by SprE (Carabetta *et al.*, 2009).

Hfq

Hfq is an RNA chaperone known to be involved in the stabilization and/or degradation of many RNAs. This widespread and highly abundant post-transcriptional regulator belongs to the Sm/Lsm family of RNA binding proteins. The active form of Hfq has a doughnut-shape homohexameric ring structure that displays the highest affinity for short single-stranded stretches of adenines and uridines adjacent to stem-loop structures (Kajitani *et al.*, 1994). The protein has three RNA-binding sites: the proximal site, which preferably binds to the 3'-hydroxyl group of the sRNA composed by the uridine-rich terminator (Otaka *et al.*, 2011; Sauer and Weichenrieder, 2011); the distal site, specific for poly(A) stretches (Mikulecky *et al.*, 2004); and a lateral site that is largely responsible for binding of the sRNA body (Sauer *et al.*, 2012). Therefore, the sRNA is anchored in the proximal site via its 3'-terminal uridine-rich terminator end, whereas the sRNA body wraps around the ring and is protected by the interaction with several of the lateral sites. The distal site assists mRNA targeting by the Hfq/sRNA complex (Sauer *et al.*, 2012). This protein can directly interact with PAP I and/or change its activity from distributive to processive (Hajnsdorf and Régnier, 2000; Mohanty *et al.*, 2004) (Figure 1A). Actually, Hfq targets many mRNAs for degradation by binding to their poly(A) tails and stimulating polyadenylation (Folichon *et al.*, 2003; Hajnsdorf and Régnier, 2000). Paradoxically, Hfq stabilizes sRNAs, probably because its binding protects them from ribonuclease attack as there is an overlapping of recognition sites between Hfq and RNase E (Folichon *et al.*, 2003). Hfq has also been shown to interact with

PNPase and RNase E (Mohanty and Kushner, 2011; Mohanty *et al.*, 2004; Morita *et al.*, 2005). The formation of variable ribonucleoprotein complexes between RNase E and Hfq/sRNAs specifically destabilizes the mRNA target (Morita *et al.*, 2005).

The pleiotropic phenotype of *hfq* null mutants reveals that this protein acts on several pathways of *E. coli* metabolism (Johansen *et al.*, 2006; Tsui *et al.*, 1994). In *Salmonella enterica* serovar Typhimurium, the deletion of *hfq* attenuates the ability of the model pathogen to infect mice, to invade epithelial cells, to secrete virulence factors and to survive inside cultured macrophages (Sittka *et al.*, 2007). This fact was recently confirmed using the insect model *Galleria mellonella* (Viegas *et al.*, 2012). In fact, it was shown that Hfq controls the expression of almost a fifth of all *Salmonella* genes, including the master regulator of SPI-1 invasion genes (*hilD)* and also the flagellar master regulator (*fhlDC)* (Sittka *et al.*, 2008).

Hfq is currently recognized as a key factor in regulation by sRNAs. This protein promotes sRNAs interaction with their mRNA targets (see details in the next subsection) (Viegas and Arraiano, 2008; Waters and Storz, 2009), although the precise mechanism by which Hfq brings mRNAs and sRNAs together is not completely understood. The Hfq binding may unfold or weaken RNA secondary structures, allowing sRNAs to access their targets (Geissmann and Touati, 2004; Moll *et al.*, 2003). Alternatively, the simultaneous interaction between the sRNA with the Hfq proximal and lateral sites, and the mRNA with its distal site, may raise the local concentrations of the two RNAs, thereby increasing the probability of sRNA–mRNA interaction (Link *et al.*, 2009). When the interaction between the two RNA molecules is stable, Hfq dissociates or is proteolytically removed from the complex (Moll *et al.*, 2003).

While the focus of study has long been on *E. coli* Hfq, a role in sRNA-mediated regulation extends to distant bacteria (Lybecker *et al.*, 2010; Nielsen *et*

al., 2010). Intriguingly, a growing number of bacteria such as *Burkholderia cenocepacia* are now known to contain multiple Hfq proteins (Ramos *et al.*, 2011).

RNA regulators

RNA regulators have been extensively studied over the last years because of their high importance in the post-transcriptional regulation of bacterial gene expression. They have become another important factor to consider in the global picture of RNA turnover. Their action can directly trigger the degradation of specific mRNAs, thus changing gene expression.

The mechanisms of regulation by these RNA molecules are very complex and reports of different modes of action are becoming increasingly common in the literature. Therefore, RNA regulators are divided in different classes depending on their mode of action. Some interact with a protein to modify its activity by mimicking and thus competing with RNA or DNA targets. For instance, CsrB and CsrC tightly regulate the activity of the global post-transcriptional regulator CsrA by binding and sequestering several of these proteins simultaneously (Babitzke and Romeo, 2007). Another well-known protein-binding sRNA is the *E. coli* 6S RNA which was found to tightly bind and inhibit the housekeeping form of RNA polymerase (σ^{70}-RNAP). The interaction between 6S and σ^{70} holoenzyme, which occurs mainly during stationary phase of growth, inhibits the transcription from certain σ^{70} promoters (Wassarman, 2007a; Wassarman, 2007b).

The vast majority of the small RNAs belongs to the class of antisense sRNAs that act by directly base-pairing with their mRNA targets. Antisense sRNAs can act either in *cis* or in *trans* depending on their genomic location in relation to their mRNA target(s). MicA sRNA is the only known case of a sRNA in *E. coli* which targets both in *cis* and in *trans* (Gogol *et al.*, 2011; Rasmussen *et al.*, 2005; Udekwu, 2010; Udekwu *et al.*, 2005). *Cis*-encoded sRNAs are transcribed

from the same *locus*, but in the opposite sense sharing extended regions of complete complementarity with theirs targets. The best known examples are those involved in the replication of plasmids such as the antisense RNA CopA that inhibits the replication of plasmid R1 (Givskov and Molin, 1984; Stougaard *et al.*, 1981) and the copy number regulator of the plasmid ColE1 RNAI (Lin-Chao and Cohen, 1991). Other example of *cis*-encoded sRNAs are those involved in the repression of genes that encode potentially toxic proteins (Fozo *et al.*, 2008; Gerdes and Wagner, 2007). This type of sRNAs was also found to be associated with bacteriophages and transposons and a few of them have been identified in the bacterial chromosome (for a review (Brantl, 2007)). Nevertheless, the bulk of antisense sRNAs are *trans*-encoded, being transcribed from a distinct *locus*. Almost all of the sRNAs of this class are expressed under specific growth conditions ranging from limiting iron (Fur-repressed RyhB sRNA), oxidative stress (OxyR-activated OxyS sRNA), outer membrane stress (σ^E-induced MicA and RybB sRNAs), elevated glycine (GcvA-induced GcvB sRNA), high glucose-6-phosphate concentrations (SgrR-activated SgrS sRNA) and glucose starvation (CRP-repressed Spot42 sRNA and CRP-activated CyaR sRNA) (reviewed in (Görke and Vogel, 2008; Gottesman, 2005; Papenfort and Vogel, 2009)). In contrast to the *cis*-encoded, the *trans*-encoded sRNAs display a limited and often non-contiguous target complementarity. This limited complementarity not only allows a single sRNA to act on several different targets (Papenfort and Vogel, 2009), but also consents several sRNAs to act on the same target presumably under different stress conditions (Battesti *et al.*, 2011). As the complementarity of *trans*-encoded sRNAs with the respective targets is imperfect they typically require Hfq for target interaction and/or intracellular stability. Hfq-dependent antisense regulation is widespread in Gram-negative bacteria, but only one example was reported in Gram-positive bacteria in *L. monocytogenes* (Nielsen *et*

al., 2010). The *trans*-encoded sRNAs usually exhibit a structure composed by three different domains: the first region, often called seed region, is highly conserved and is involved on the base-pairing to target RNAs; the second domain is the binding site for Hfq; and the third region comprises a structured 3′-end followed by poly(U) that promotes the Rho-independent transcription termination and protects the sRNA against 3′-exonucleases. The poly(U) region at the 3′-end can also be recognized by Hfq, possibly serving as a loading site (Otaka *et al.*, 2011; Sauer and Weichenrieder, 2011) (Figure 3A).

The result of the sRNA/target pairing can be one of the following: inhibition of translation; mRNA degradation; stimulation of translation; and stabilization of the mRNA (Gottesman and Storz, 2011). Despites few cases are known, examples of gene expression activation by sRNAs were reported, in many cases by preventing or overcoming the formation of an inhibitory secondary structure. For instance, the translation of the *rpoS* mRNA is positively regulated by the action of the sRNAs DsrA, RprA, and ArcZ (Majdalani *et al.*, 1998; Majdalani *et al.*, 2002; Soper *et al.*, 2010). However, the majority of these *trans*-encoded sRNAs usually act to repress translation and/or accelerate the mRNA degradation. Binding of the sRNA usually sequesters the ribosome binding site and, consequently, prevents the 30S ribosome loading. Following translational repression, the mRNA target often becomes substrate for RNase E or RNase III (Caron *et al.*, 2010; Massé *et al.*, 2003b). This initial cleavage occurs not only in the vicinity of the sRNA/mRNA interaction (Pfeiffer *et al.*, 2009; Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005) but also at distal sites downstream of the interaction (Prévost *et al.*, 2011). Interestingly, the RNA degradosome was shown to interact with the 70S ribosome and also with polysomes (Tsai *et al.*, 2012). This recent discovery led to the proposition that the degradosome may rest "passively" on a ribosome, moving from one to the next as they move along in a polysome assembly. When a sRNA/Hfq complex forms on the emerging 5′-end of the

transcript, it could be bound by the "passive" degradosome assembly. In this position, the degradosome is poised to trigger cleavage of transcripts as they emerge from the end of the polysome. The action of sRNAs is, however, not limited to the RBS. Recognition of the target upstream of its 5'-UTR (Darfeuille *et al.*, 2007; Sharma *et al.*, 2007; Vecerek *et al.*, 2007) and binding inside the coding sequence of the mRNA target (Bouvier *et al.*, 2008; Papenfort *et al.*, 2010; Pfeiffer *et al.*, 2009) has also been reported (Figure 3B).

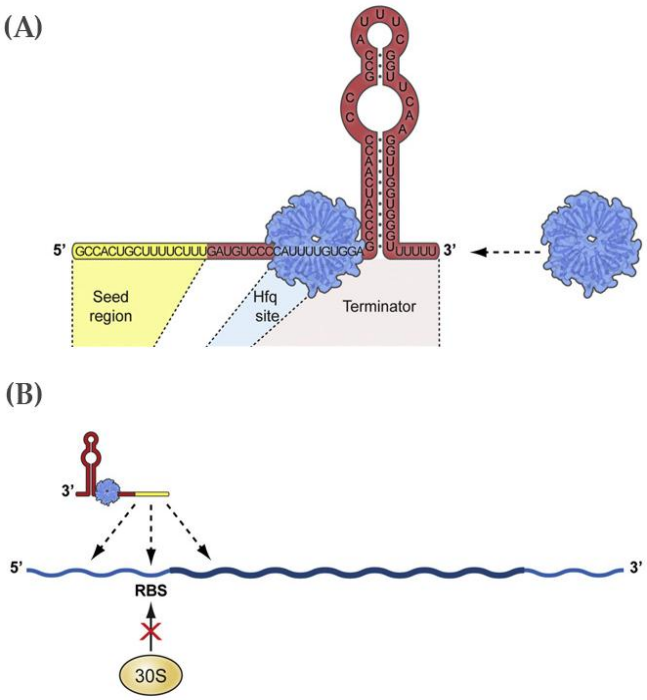

FIGURE 3 – Properties of *trans*-encoded base-pairing sRNAs. A) Diagram showing the structure of Hfq-binding sRNAs. **B)** Diagram showing the different positions at which sRNAs can block ribosome binding. Adapted from (Storz *et al.*, 2011).

In addition to the protein-binding and to the base-pairing sRNAs there are two other types of RNA regulators, the riboswitches and CRISPRs sRNAs. The riboswitches are sequences usually found within the 5'-UTR of the mRNA

that they regulate and which can adopt different conformations in response to environmental signals. These include stalled ribosomes, uncharged tRNAs, elevated temperatures and/or small molecule ligands (for a review (Breaker, 2012; Smith *et al.*, 2010)). The more recently discovered group of RNA regulators, CRISPR RNAs, function as a prokaryotic immune system, in that they provide resistance to exogenous genetic elements such as plasmids and bacteriophages (Bhaya *et al.*, 2011; van der Oost *et al.*, 2009).

The different types of small RNA molecules serve as diverse regulators of gene expression that impact almost every aspect of bacterial physiology. Accordingly, the study of these RNAs is a crucial step to understand the gene regulation in bacteria.

REGULATION OF RNASES

As effectors that rapidly modulate the levels of RNAs, the expression of RNases must be tightly regulated in a process that responds to many different signals and which may relevantly affect RNA turnover rates. This regulation is known to occur in several ways. RNases can regulate their own levels (auto-regulation) and/or be regulated by other ribonucleases (cross-regulation). Additionally, their intracellular bulk level can be affected by the medium composition and other environmental factors that alter the cell growth rate. These and other approaches of regulation of the degradative ribonucleolytic machinery in prokaryotes are discussed below in further detail.

Auto-regulation and cross-regulation

Both RNase E (Jain and Belasco, 1995) and RNase III (Bardwell *et al.*, 1989) have the ability to control the decay of their own mRNAs, thereby regulating their own expression and maintaining the enzyme levels within a narrow range.

In both cases, a cleavage that occurs in the 5'-UTR region of the mRNA promotes their decay. PNPase is able to regulate its own expression as well, but in an RNase III-dependent manner (Portier *et al.*, 1987). PNPase acts in concert with RNase III to degrade a double-stranded region in the 5'-end of *pnp* mRNA. The removal of this region impairs translation and allows further degradation of *pnp* transcript by RNase E (Carzaniga *et al.*, 2009). PNPase expression is also regulated by RNase II (Zilhão *et al.*, 1996a). This latter case constitutes an interesting example of cross-regulation: in the absence of RNase II, PNPase levels are increased, while PNPase overexpression leads to a decrease in RNase II activity. PNPase controls RNase II activity by degrading its mRNA (Zilhão *et al.*, 1996a). RNase III and RNase E endonucleases are also involved in the control of RNase II expression (Zilhão *et al.*, 1995b). While RNase III regulates RNase II by affecting PNPase levels, RNase E directly intervenes in the degradation of *rnb* mRNA. RNase E is also the main enzyme responsible for the processing of *rnr* transcripts encoding RNase R (Cairrão and Arraiano, 2006).

Regulation by environmental conditions and other cellular modulations

RNase E is responsible for many processes of RNA decay and maturation. Therefore, the enzyme must be tightly regulated because any change in the level of its expression has important cellular repercussions. As discussed above, the 5'-end phosphorylation state, folding and translation of a given mRNA substrate can modulate the RNase E cleavage efficiency by altering the enzyme's accessibility to the transcript. In addition, RraA and RraB (regulators of RNase activity A and B, respectively), interact with RNase E to inhibit its activity (Gao *et al.*, 2006; Lee *et al.*, 2003). In addition to these factors, environmental conditions such as temperature and medium composition have been reported to directly

affect the activity and cellular concentration of RNase E (Barlow *et al.*, 1998; Georgellis *et al.*, 1992; Le Derout *et al.*, 2002). As a consequence, the RNA processing and stability of specific transcripts is affected (Barlow *et al.*, 1998; Georgellis *et al.*, 1992; Le Derout *et al.*, 2002).

RNase R is a ribonuclease whose levels also change in response to different environmental stimuli (Chen and Deutscher, 2005). These include the entry into stationary phase (2-fold), heat-shock (~2-fold), and cold-shock (7–8 fold) (Andrade *et al.*, 2006; Cairrão *et al.*, 2003; Chen and Deutscher, 2005). From the different stress conditions analyzed, cold-shock treatment results by far in the highest up-regulation effect over RNase R. This marked increase in RNase R levels is probably related to its ability to overcome RNA secondary structures, whose formation is thermodynamically favoured under low temperatures. Northern blot analysis of the transcript's decay has indicated an increase in *rnr* stability during cold-shock (Cairrão *et al.*, 2003). Moreover, Western blot analysis of RNase R degradation after translational arrest has revealed that the protein itself is highly stabilized under cold-shock, stationary-phase and growth in minimal medium (Liang and Deutscher, 2010). It has been shown that the tmRNA–SmpB system can be responsible for the low stability of the RNase R protein in exponential-phase cells, through the interaction with the region encompassing the S1 domain and the C-terminus of the exonuclease (Liang and Deutscher, 2010). This process was recently elucidated by the finding that a post-translational modification of RNase R, that specifically occurs in exponential phase, leads to a tighter binding of tmRNA and SmpB (Liang and Deutscher, 2012a; Liang *et al.*, 2011). These two later components stimulate the binding of two proteases (Lon and HslUV) to the N-terminal region of RNase R promoting its degradation (Liang and Deutscher, 2012b). Albeit to a lower level, growth at low temperatures also induces PNPase expression, which is an essential enzyme in this condition (Piazza *et al.*, 1996; Zangrossi *et al.*, 2000). Moreover, certain

mutations of the PNPase RNA binding domains have been shown to confer a cold-sensitive phenotype (Briani *et al.*, 2007; García-Mena *et al.*, 1999; Matus-Ortega *et al.*, 2007).

Like RNase R, RNase II stability is post-translationally regulated. Deletion of *gmr* (gene modulating RNase II), a gene that lies just downstream of *rnb*, causes the accumulation of this exonuclease by increasing the protein stability more than 2-fold (Cairrão *et al.*, 2001). RNase II is also more abundant in rich medium compared to minimal medium and is sensitive to the nitrogen content of the medium. This regulation is abolished in a *gmr* mutant (Cairrão *et al.*, 2001). One possible explanation is that the PAS domain of Gmr protein can act as a sensor, which monitors nutrients in the growth medium and carries the signals to the proteolytic enzymes responsible for RNase II degradation. Interestingly, beyond being regulated post-transcriptionally (at the level of mRNA and protein), RNase II has two promoters and its expression is also controlled at the transcriptional level (Zilhão *et al.*, 1995a; Zilhão *et al.*, 1993; Zilhão *et al.*, 1996b).

Polyadenylation is implicated in the destabilization of a variety of transcripts but it also arises as a factor controlling RNases levels in the cell. A high intracellular level of poly(A) tails stabilizes *pnp* and *rne* transcripts, thereby leading to increased PNPase and RNase E levels, respectively (Mohanty and Kushner, 2002).

The activity of RNases represents a checkpoint on RNA regulation. Accordingly, their expression has to be tightly regulated in different ways, as small variations on their levels may have a tremendous impact on global RNA decay.

CONCLUDING REMARKS

RNA degradation is an intricate mechanism and a process that plays a fundamental role in the regulation of gene expression. The steady-state level of a given transcript is dependent on a high level of coordination between the different players involved in transcription and degradation. This complex network permits a rapid response to challenging conditions, and it is therefore not surprising that RNA degradation is not exclusively deterministic but is also controlled by external stimuli. RNA decay is a major link in the chain of bacterial adaptation, a key for survival and development. In this chapter, I aimed at providing an up-to-date picture of RNA stability/decay and its control in prokaryotes. It involves numerous players and relies on several features. However, the main effectors are ribonucleases, whose diversity, structures, targets, and modes of action can vary significantly, providing multiple solutions for a similar issue. Ribonucleases can act independently, or in a concerted way, as well as in higher order protein complexes such as the degradosome. Their activity can be modulated by regulatory proteins. Besides this regulatory network, the fate of an RNA molecule is intimately related to its sequence and structural features. Indeed, the characteristics of an mRNA molecule can determine ribosomal pausing, targeting by sRNAs, and also elect which RNases will be able to act.

Novel players that influence RNA stability are still being discovered. These new findings not only increase our knowledge of this topic but often challenge the conventional models. For instance, the finding of the RNA pyrophosphohydrolase RppH in *E. coli* revealed an alternative pathway for degradation. The homologue in *B. subtilis* was just recently proposed (Richards *et al.*, 2011). The ability of the RNase J enzymes to cleave RNA in the 5′–3′ direction has also restructured the conventional view of RNA decay in prokaryotes.

Another example is the discovery of sRNAs as essential regulators of mRNA stability, which has significantly altered the established models of RNA turnover. Degradation of these small molecules is in turn directed by RNases and other factors, namely polyadenylation. Their degradation can alter the concentration of the target mRNA by directly modifying the cellular concentration of the sRNA.

There are also indications of the existence of a cellular compartmentalization in bacteria, which could help to synchronize regulatory events. Certain mRNAs have been shown to migrate to particular cellular domains where their future protein products are required (Nevo-Dinur *et al.*, 2011). In another study the mRNAs studied exhibited limited dispersion from their site of transcription. In this latter case the chromosome was proposed to be a spatial organizer of mRNA and related processes (Montero Llopis *et al.*, 2010). Different mRNAs were studied and this may account for these apparently divergent observations. The identification of additional players and regulatory processes is essential for fully understanding RNA decay. Although there has been tremendous progress in our understanding of the posttranscriptional control of RNA stability and decay, the continuing discovery of new processes illustrates that the intricacies of RNA degradation are still far from being completely understood, and a remarkable amount remains to be learned.

AIM OF THIS DISSERTATION

This dissertation is mainly focused on the involvement of two small non-coding RNAs, MicA and SraL, in the post-transcriptional regulation of gene expression in *Salmonella* Typhimurium.

MicA is one of the best and most extensively studied sRNAs in Gram-negative bacteria. It was firstly identified in a global *E. coli* sRNA screen, and observed to accumulate as a small transcript (~70 nt) when cells stopped growing (Argaman *et al.*, 2001). Afterwards, MicA was also detected in *Salmonella* cells in the same conditions (Figueroa-Bossi *et al.*, 2006; Papenfort *et al.*, 2006; Viegas *et al.*, 2007). We have previously studied the influence of several RNases and also PAP I in the stability of MicA (Viegas *et al.*, 2007). The stability of this sRNA was shown to be mainly affected by PNPase, RNase III and RNase E through the degradosome complex. In the first part of this Doctoral work we aimed to investigate more deeply the contribution of the two main *Salmonella* endoribonucleases in the decay of this sRNA. To this purpose, we studied the degradation of MicA in mutants for these RNases. We have expressed and purified both RNase E and RNase III from *Salmonella* and studied the direct contributions of these RNases in the degradation of this sRNA. Two targets have been described in *Salmonella* for MicA, *ompA* and *lamB* mRNAs (Bossi and Figueroa-Bossi, 2007; Papenfort *et al.*, 2006). Therefore, the same experiments were also performed using MicA coupled with its targets, *lamB* and *ompA* mRNAs.

In the second and third part of this Doctoral work we intended to study the role of SraL in *Salmonella* Typhimurium. This sRNA was firstly identified in *E. coli* in the same global screen as MicA (Argaman *et al.*, 2001). Afterwards, SraL was also identified in *Salmonella* cells in late stationary phase of growth (Viegas *et al.*, 2007). We have shown that this sRNAs is post-transcriptionally regulated by

PNPase and also by the degradosome. However, the greatest effect in the SraL stability was observed when using the PAP I mutant strain (Viegas *et al.*, 2007). Since we already had studied the post-transcriptional regulation of this sRNA one of our goals was to study how it is transcriptionally regulated. To this purpose, we performed the analysis of the SraL promoter sequence and investigated our predictions through several experimental approaches. Since the biological function of this sRNA was not described yet, our second aim was to identify its targets. In order to address this question we performed global proteomic and bioinformatic analyses. We then validated the results obtained with these approaches by Northern blot and RT-PCR experiments.

In summary, the work developed in this Dissertation aimed to deeply understand the role of two important sRNAs in *Salmonella* Typhimurium. MicA sRNA had already been shown to be a very important regulator in Gram-negative bacteria. The work developed during this Dissertation reveals more details about the post-transcriptional regulation of MicA and its targets. Moreover, it also discloses the importance of the expression of SraL in the cell, so far unknown, by revealing how its expression is triggered and also one of the biological functions of this sRNA.

REFERENCES

Amblar, M., and Arraiano, C.M. 2005. A single mutation in *Escherichia coli* ribonuclease II inactivates the enzyme without affecting RNA binding. *FEBS J*. 272:363-374.

Amblar, M., Barbas, A., Fialho, A.M., and Arraiano, C.M. 2006. Characterization of the functional domains of *Escherichia coli* RNase II. *J Mol Biol*. 360:921-933.

Amblar, M., Barbas, A., Gomez-Puertas, P., and Arraiano, C.M. 2007. The role of the S1 domain in exoribonucleolytic activity: substrate specificity and multimerization. *RNA*. 13:317-327.

Anderson, K.L., Roberts, C., Disz, T., Vonstein, V., Hwang, K., Overbeek, R., Olson, P.D., Projan, S.J., and Dunman, P.M. 2006. Characterization of the *Staphylococcus aureus* heat shock, cold shock, stringent, and SOS responses and their effects on log-phase mRNA turnover. *J Bacteriol*. 188:6739-6756.

Andrade, J.M., Cairrão, F., and Arraiano, C.M. 2006. RNase R affects gene expression in stationary phase: regulation of *ompA*. *Mol Microbiol*. 60:219-228.

Andrade, J.M., Pobre, V., Silva, I.J., Domingues, S., and Arraiano, C.M. 2009. The role of 3'-5' exoribonucleases in RNA degradation. *Prog Mol Biol Transl Sci*. 85:187-229.

Argaman, L., Hershberg, R., Vogel, J., Bejerano, G., Wagner, E.G., Margalit, H., and Altuvia, S. 2001. Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli*. *Curr Biol*. 11:941-950.

Arraiano, C.M., Andrade, J.M., Domingues, S., Guinote, I.B., Malecki, M., Matos, R.G., Moreira, R.N., Pobre, V., Reis, F.P., Saramago, M., Silva, I.J., and Viegas, S.C. 2010. The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol Rev*. 34:883-923.

Arraiano, C.M., Yancey, S.D., and Kushner, S.R. 1988. Stabilization of discrete mRNA breakdown products in *ams pnp rnb* multiple mutants of *Escherichia coli* K-12. *J Bacteriol*. 170:4625-4633.

August, J.T., Ortiz, P.J., and Hurwitz, J. 1962. Ribonucleic acid-dependent ribonucleotide incorporation. I. Purification and properties of the enzyme. *J Biol Chem*. 237:3786-3793.

Awano, N., Rajagopal, V., Arbing, M., Patel, S., Hunt, J., Inouye, M., and Phadtare, S. 2010. *Escherichia coli* RNase R has dual activities, helicase and RNase. *J Bacteriol*. 192:1344-1352.

Babitzke, P., Granger, L., Olszewski, J., and Kushner, S.R. 1993. Analysis of mRNA decay and rRNA processing in *Escherichia coli* multiple mutants carrying a deletion in RNase III. *J Bacteriol*. 175:229-239.

Babitzke, P., and Romeo, T. 2007. CsrB sRNA family: sequestration of RNA-binding regulatory proteins. *Curr Opin Microbiol*. 10:156-163.

Baker, K.E., and Mackie, G.A. 2003. Ectopic RNase E sites promote bypass of 5'-end-dependent mRNA decay in *Escherichia coli*. *Mol Microbiol*. 47:75-88.

Barbas, A., Matos, R.G., Amblar, M., López-Viñas, E., Gomez-Puertas, P., and Arraiano, C.M. 2008. New insights into the mechanism of RNA degradation by ribonuclease II: identification of the residue responsible for setting the RNase II end product. *J Biol Chem*. 283:13070-13076.

Barbas, A., Matos, R.G., Amblar, M., López-Viñas, E., Gomez-Puertas, P., and Arraiano, C.M. 2009. Determination of key residues for catalysis and RNA cleavage specificity: one mutation turns RNase II into a "SUPER-ENZYME". *J Biol Chem*. 284:20486-20498.

Bardwell, J.C., Régnier, P., Chen, S.M., Nakamura, Y., Grunberg-Manago, M., and Court, D.L. 1989. Autoregulation of RNase III operon by mRNA processing. *EMBO J*. 8:3401-3407.

Barlow, T., Berkmen, M., Georgellis, D., Bayr, L., Arvidson, S., and von Gabain, A. 1998. RNase E, the major player in mRNA degradation, is down-regulated in *Escherichia coli* during a transient growth retardation (diauxic lag). *Biol Chem*. 379:33-38.

Battesti, A., Majdalani, N., and Gottesman, S. 2011. The RpoS-mediated general stress response in *Escherichia coli*. *Annu Rev Microbiol*. 65:189-213.

Bechhofer, D.H. 2009. Messenger RNA decay and maturation in *Bacillus subtilis*. *Prog Mol Biol Transl Sci*. 85:231-273.

Bernstein, J.A., Khodursky, A.B., Lin, P.H., Lin-Chao, S., and Cohen, S.N. 2002. Global analysis of mRNA decay and abundance in *Escherichia coli* at single-gene resolution using two-color fluorescent DNA microarrays. *Proc Natl Acad Sci U S A*. 99:9697-9702.

Bhaya, D., Davison, M., and Barrangou, R. 2011. CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annu Rev Genet*. 45:273-297.

Blaszczyk, J., Gan, J., Tropea, J.E., Court, D.L., Waugh, D.S., and Ji, X. 2004. Noncatalytic assembly of ribonuclease III with double-stranded RNA. *Structure*. 12:457-466.

Bossi, L., and Figueroa-Bossi, N. 2007. A small RNA downregulates LamB maltoporin in *Salmonella*. *Mol Microbiol*. 65:799-810.

Bouvier, M., Sharma, C.M., Mika, F., Nierhaus, K.H., and Vogel, J. 2008. Small RNA binding to 5' mRNA coding region inhibits translational initiation. *Mol Cell*. 32:827-837.

Bralley, P., Gust, B., Chang, S., Chater, K.F., and Jones, G.H. 2006. RNA 3'-tail synthesis in *Streptomyces*: *in vitro* and *in vivo* activities of RNase PH, the

SCO3896 gene product and polynucleotide phosphorylase. *Microbiology*. 152:627-636.

Brantl, S. 2007. Regulatory mechanisms employed by *cis*-encoded antisense RNAs. *Curr Opin Microbiol*. 10:102-109.

Breaker, R.R. 2012. Riboswitches and the RNA world. *Cold Spring Harb Perspect Biol*. 4.

Briani, F., Del Favero, M., Capizzuto, R., Consonni, C., Zangrossi, S., Greco, C., De Gioia, L., Tortora, P., and Dehò, G. 2007. Genetic analysis of polynucleotide phosphorylase structure and functions. *Biochimie*. 89:145-157.

Cairrão, F., and Arraiano, C.M. 2006. The role of endoribonucleases in the regulation of RNase R. *Biochem Biophys Res Commun*. 343:731-737.

Cairrão, F., Chora, A., Zilhão, R., Carpousis, A.J., and Arraiano, C.M. 2001. RNase II levels change according to the growth conditions: characterization of *gmr*, a new *Escherichia coli* gene involved in the modulation of RNase II. *Mol Microbiol*. 39:1550-1561.

Cairrão, F., Cruz, A., Mori, H., and Arraiano, C.M. 2003. Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. *Mol Microbiol*. 50:1349-1360.

Callaghan, A.J., Marcaida, M.J., Stead, J.A., McDowall, K.J., Scott, W.G., and Luisi, B.F. 2005. Structure of *Escherichia coli* RNase E catalytic domain and implications for RNA turnover. *Nature*. 437:1187-1191.

Campos-Guillén, J., Bralley, P., Jones, G.H., Bechhofer, D.H., and Olmedo-Alvarez, G. 2005. Addition of poly(A) and heteropolymeric 3' ends in *Bacillus subtilis* wild-type and polynucleotide phosphorylase-deficient strains. *J Bacteriol*. 187:4698-4706.

Cao, G.J., and Sarkar, N. 1992. Identification of the gene for an *Escherichia coli* poly(A) polymerase. *Proc Natl Acad Sci U S A*. 89:10380-10384.

Cao, G.J., and Sarkar, N. 1997. Stationary phase-specific mRNAs in *Escherichia coli* are polyadenylated. *Biochem Biophys Res Commun*. 239:46-50.

Carabetta, V.J., Mohanty, B.K., Kushner, S.R., and Silhavy, T.J. 2009. The response regulator SprE (RssB) modulates polyadenylation and mRNA stability in *Escherichia coli*. *J Bacteriol*. 191:6812-6821.

Carabetta, V.J., Silhavy, T.J., and Cristea, I.M. 2010. The response regulator SprE (RssB) is required for maintaining poly(A) polymerase I-degradosome association during stationary phase. *J Bacteriol*. 192:3713-3721.

Caron, M.P., Lafontaine, D.A., and Massé, E. 2010. Small RNA-mediated regulation at the level of transcript stability. *RNA Biol*. 7:140-144.

Carpousis, A.J., Luisi, B.F., and McDowall, K.J. 2009. Endonucleolytic initiation of mRNA decay in *Escherichia coli*. *Prog Mol Biol Transl Sci*. 85:91-135.

Carpousis, A.J., Van Houwe, G., Ehretsmann, C., and Krisch, H.M. 1994. Copurification of *E. coli* RNAase E and PNPase: evidence for a specific association between two enzymes important in RNA processing and degradation. *Cell*. 76:889-900.

Carzaniga, T., Briani, F., Zangrossi, S., Merlino, G., Marchi, P., and Dehò, G. 2009. Autogenous regulation of *Escherichia coli* polynucleotide phosphorylase expression revisited. *J Bacteriol*. 191:1738-1748.

Charpentier, X., Faucher, S.P., Kalachikov, S., and Shuman, H.A. 2008. Loss of RNase R induces competence development in *Legionella pneumophila*. *J Bacteriol*. 190:8126-8136.

Chen, C., and Deutscher, M.P. 2005. Elevation of RNase R in response to multiple stress conditions. *J Biol Chem*. 280:34393-34396.

Cheng, Z.F., and Deutscher, M.P. 2003. Quality control of ribosomal RNA mediated by polynucleotide phosphorylase and RNase R. *Proc Natl Acad Sci U S A*. 100:6388-6393.

Commichau, F.M., Rothe, F.M., Herzberg, C., Wagner, E., Hellwig, D., Lehnik-Habrink, M., Hammer, E., Völker, U., and Stülke, J. 2009. Novel activities of glycolytic enzymes in *Bacillus subtilis*: interactions with essential proteins involved in mRNA processing. *Mol Cell Proteomics*. 8:1350-1360.

Condon, C. 2003. RNA processing and degradation in *Bacillus subtilis*. *Microbiol Mol Biol Rev*. 67:157-174, table of contents.

Condon, C. 2010. What is the role of RNase J in mRNA turnover? *RNA Biol*. 7:316-321.

Dam Mikkelsen, N., and Gerdes, K. 1997. Sok antisense RNA from plasmid R1 is functionally inactivated by RNase E and polyadenylated by poly(A) polymerase I. *Mol Microbiol*. 26:311-320.

Darfeuille, F., Unoson, C., Vogel, J., and Wagner, E.G. 2007. An antisense RNA inhibits translation by competing with standby ribosomes. *Mol Cell*. 26:381-392.

de la Sierra-Gallay, I.L., Zig, L., Jamalli, A., and Putzer, H. 2008. Structural insights into the dual activity of RNase J. *Nat Struct Mol Biol*. 15:206-212.

Deana, A., Celesnik, H., and Belasco, J.G. 2008. The bacterial enzyme RppH triggers messenger RNA degradation by 5' pyrophosphate removal. *Nature*. 451:355-358.

Deutscher, M.P., and Reuven, N.B. 1991. Enzymatic basis for hydrolytic versus phosphorolytic mRNA degradation in *Escherichia coli* and *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA*. 88:3277-3280.

Domingues, S., Matos, R.G., Reis, F.P., Fialho, A.M., Barbas, A., and Arraiano, C.M. 2009. Biochemical characterization of the RNase II family of

exoribonucleases from the human pathogens *Salmonella typhimurium* and *Streptococcus pneumoniae*. *Biochemistry*. 48:11848-11857.

Dorléans, A., Li de la Sierra-Gallay, I., Piton, J., Zig, L., Gilet, L., Putzer, H., and Condon, C. 2011. Molecular basis for the recognition and cleavage of RNA by the bifunctional 5'-3' exo/endoribonuclease RNase J. *Structure*. 19:1252-1261.

Dreyfus, M., and Régnier, P. 2002. The poly(A) tail of mRNAs: bodyguard in eukaryotes, scavenger in bacteria. *Cell*. 111:611-613.

Durand, S., Gilet, L., Bessières, P., Nicolas, P., and Condon, C. 2012. Three essential ribonucleases - RNase Y, J1, and III - control the abundance of a majority of *Bacillus subtilis* mRNAs. *PLoS Genet*. 8:e1002520.

Even, S., Pellegrini, O., Zig, L., Labas, V., Vinh, J., Bréchemmier-Baey, D., and Putzer, H. 2005. Ribonucleases J1 and J2: two novel endoribonucleases in *B.subtilis* with functional homology to *E.coli* RNase E. *Nucleic Acids Res*. 33:2141-2152.

Falaleeva, M.V., Chetverina, H.V., Ugarov, V.I., Uzlova, E.A., and Chetverin, A.B. 2008. Factors influencing RNA degradation by *Thermus thermophilus* polynucleotide phosphorylase. *FEBS J*. 275:2214-2226.

Fang, M., Zeisberg, W.M., Condon, C., Ogryzko, V., Danchin, A., and Mechold, U. 2009. Degradation of nanoRNA is performed by multiple redundant RNases in *Bacillus subtilis*. *Nucleic Acids Res*. 37:5114-5125.

Feng, Y., and Cohen, S.N. 2000. Unpaired terminal nucleotides and 5' monophosphorylation govern 3' polyadenylation by *Escherichia coli* poly(A) polymerase I. *Proc Natl Acad Sci U S A*. 97:6415-6420.

Figueroa-Bossi, N., Lemire, S., Maloriol, D., Balbontin, R., Casadesus, J., and Bossi, L. 2006. Loss of Hfq activates the sigmaE-dependent envelope stress response in *Salmonella enterica*. *Mol Microbiol*. 62:838-852.

Folichon, M., Arluison, V., Pellegrini, O., Huntzinger, E., Régnier, P., and Hajnsdorf, E. 2003. The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation. *Nucleic Acids Res*. 31:7302-7310.

Fozo, E.M., Hemm, M.R., and Storz, G. 2008. Small toxic proteins and the antisense RNAs that repress them. *Microbiol Mol Biol Rev*. 72:579-589, Table of Contents.

Frazão, C., McVey, C.E., Amblar, M., Barbas, A., Vonrhein, C., Arraiano, C.M., and Carrondo, M.A. 2006. Unravelling the dynamics of RNA degradation by ribonuclease II and its RNA-bound complex. *Nature*. 443:110-114.

Gan, J., Shaw, G., Tropea, J.E., Waugh, D.S., Court, D.L., and Ji, X. 2008. A stepwise model for double-stranded RNA processing by ribonuclease III. *Mol Microbiol*. 67:143-154.

Gan, J., Tropea, J.E., Austin, B.P., Court, D.L., Waugh, D.S., and Ji, X. 2006. Structural insight into the mechanism of double-stranded RNA processing by ribonuclease III. *Cell*. 124:355-366.

Gao, J., Lee, K., Zhao, M., Qiu, J., Zhan, X., Saxena, A., Moore, C.J., Cohen, S.N., and Georgiou, G. 2006. Differential modulation of *E. coli* mRNA abundance by inhibitory proteins that alter the composition of the degradosome. *Mol Microbiol*. 61:394-406.

García-Martínez, J., Aranda, A., and Pérez-Ortín, J.E. 2004. Genomic run-on evaluates transcription rates for all yeast genes and identifies gene regulatory mechanisms. *Mol Cell*. 15:303-313.

García-Mena, J., Das, A., Sánchez-Trujillo, A., Portier, C., and Montañez, C. 1999. A novel mutation in the KH domain of polynucleotide phosphorylase affects autoregulation and mRNA decay in *Escherichia coli*. *Mol Microbiol*. 33:235-248.

Garza-Sánchez, F., Shoji, S., Fredrick, K., and Hayes, C.S. 2009. RNase II is important for A-site mRNA cleavage during ribosome pausing. *Mol Microbiol*. 73:882-897.

Ge, Z., Mehta, P., Richards, J., and Karzai, A.W. 2010. Non-stop mRNA decay initiates at the ribosome. *Mol Microbiol*. 78:1159-1170.

Geissmann, T.A., and Touati, D. 2004. Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. *EMBO J*. 23:396-405.

Georgellis, D., Arvidson, S., and von Gabain, A. 1992. Decay of *ompA* mRNA and processing of 9S RNA are immediately affected by shifts in growth rate, but in opposite manners. *J Bacteriol*. 174:5382-5390.

Gerdes, K., and Wagner, E.G. 2007. RNA antitoxins. *Curr Opin Microbiol*. 10:117-124.

Ghosh, S., and Deutscher, M.P. 1999. Oligoribonuclease is an essential component of the mRNA decay pathway. *Proc Natl Acad Sci U S A*. 96:4372-4377.

Givskov, M., and Molin, S. 1984. Copy mutants of plasmid R1: effects of base pair substitutions in the *copA* gene on the replication control system. *Mol Gen Genet*. 194:286-292.

Gogol, E.B., Rhodius, V.A., Papenfort, K., Vogel, J., and Gross, C.A. 2011. Small RNAs endow a transcriptional activator with essential repressor functions for single-tier control of a global stress regulon. *Proc Natl Acad Sci U S A*. 108:12875-12880.

Goodrich, A.F., and Steege, D.A. 1999. Roles of polyadenylation and nucleolytic cleavage in the filamentous phage mRNA processing and decay pathways in *Escherichia coli*. *RNA*. 5:972-985.

Gopalakrishna, Y., Langley, D., and Sarkar, N. 1981. Detection of high levels of polyadenylate-containing RNA in bacteria by the use of a single-step RNA isolation procedure. *Nucleic Acids Res*. 9:3545-3554.

Görke, B., and Vogel, J. 2008. Noncoding RNA control of the making and breaking of sugars. *Genes Dev*. 22:2914-2925.

Gottesman, S. 2005. Micros for microbes: non-coding regulatory RNAs in bacteria. *Trends Genet*. 21:399-404.

Gottesman, S., and Storz, G. 2011. Bacterial small RNA regulators: versatile roles and rapidly evolving variations. *Cold Spring Harb Perspect Biol*. 3.

Grigull, J., Mnaimneh, S., Pootoolal, J., Robinson, M.D., and Hughes, T.R. 2004. Genome-wide analysis of mRNA stability using transcription inhibitors and microarrays reveals posttranscriptional control of ribosome biogenesis factors. *Mol Cell Biol*. 24:5534-5547.

Hajnsdorf, E., and Régnier, P. 2000. Host factor Hfq of *Escherichia coli* stimulates elongation of poly(A) tails by poly(A) polymerase I. *Proc Natl Acad Sci U S A*. 97:1501-1505.

Hambraeus, G., von Wachenfeldt, C., and Hederstedt, L. 2003. Genome-wide survey of mRNA half-lives in *Bacillus subtilis* identifies extremely stable mRNAs. *Mol Genet Genomics*. 269:706-714.

Hardwick, S.W., Gubbey, T., Hug, I., Jenal, U., and Luisi, B.F. 2012. Crystal structure of *Caulobacter crescentus* polynucleotide phosphorylase reveals a mechanism of RNA substrate channelling and RNA degradosome assembly. *Open Biol*. 2:120028.

Haugel-Nielsen, J., Hajnsdorf, E., and Régnier, P. 1996. The *rpsO* mRNA of *Escherichia coli* is polyadenylated at multiple sites resulting from endonucleolytic processing and exonucleolytic degradation. *EMBO J*. 15:3144-3152.

Herskovitz, M.A., and Bechhofer, D.H. 2000. Endoribonuclease RNase III is essential in *Bacillus subtilis*. *Mol Microbiol*. 38:1027-1033.

Houseley, J., and Tollervey, D. 2009. The many pathways of RNA degradation. *Cell*. 136:763-776.

Hunt, A., Rawlins, J.P., Thomaides, H.B., and Errington, J. 2006. Functional analysis of 11 putative essential genes in *Bacillus subtilis*. *Microbiology*. 152:2895-2907.

Hutchison, C.A., Peterson, S.N., Gill, S.R., Cline, R.T., White, O., Fraser, C.M., Smith, H.O., and Venter, J.C. 1999. Global transposon mutagenesis and a minimal *Mycoplasma* genome. *Science*. 286:2165-2169.

Hutzinger, R., Mrazek, J., Vorwerk, S., and Huttenhofer, A. 2010. NcRNA-microchip analysis: A novel approach to identify differential expression of non-coding RNAs. *RNA Biol*. 7:81-90.

Jain, C., and Belasco, J.G. 1995. RNase E autoregulates its synthesis by controlling the degradation rate of its own mRNA in *Escherichia coli*: unusual sensitivity of the *rne* transcript to RNase E activity. *Genes Dev*. 9:84-96.

Janga, S.C., and Babu, M.M. 2009. Transcript stability in the protein interaction network of *Escherichia coli*. *Mol Biosyst*. 5:154-162.

Jarrige, A., Bréchemier-Baey, D., Mathy, N., Duché, O., and Portier, C. 2002. Mutational analysis of polynucleotide phosphorylase from *Escherichia coli*. *J Mol Biol*. 321:397-409.

Jasiecki, J., and Wegrzyn, G. 2005. Localization of *Escherichia coli* poly(A) polymerase I in cellular membrane. *Biochem Biophys Res Commun*. 329:598-602.

Jaskiewicz, L., and Filipowicz, W. 2008. Role of Dicer in posttranscriptional RNA silencing. *Curr Top Microbiol Immunol*. 320:77-97.

Johansen, J., Rasmussen, A.A., Overgaard, M., and Valentin-Hansen, P. 2006. Conserved small non-coding RNAs that belong to the sigmaE regulon: role in down-regulation of outer membrane proteins. *J Mol Biol*. 364:1-8.

Kaberdin, V.R., Miczak, A., Jakobsen, J.S., Lin-Chao, S., McDowall, K.J., and von Gabain, A. 1998. The endoribonucleolytic N-terminal half of *Escherichia coli* RNase E is evolutionarily conserved in *Synechocystis* sp. and other bacteria but not the C-terminal half, which is sufficient for degradosome assembly. *Proc Natl Acad Sci U S A*. 95:11637-11642.

Kajitani, M., Kato, A., Wada, A., Inokuchi, Y., and Ishihama, A. 1994. Regulation of the *Escherichia coli* hfq gene encoding the host factor for phage Q beta. *J Bacteriol*. 176:531-534.

Karzai, A.W., and Sauer, R.T. 2001. Protein factors associated with the SsrA.SmpB tagging and ribosome rescue complex. *Proc Natl Acad Sci U S A*. 98:3040-3044.

Keiler, K.C. 2008. Biology of trans-translation. *Annu Rev Microbiol*. 62:133-151.

Khemici, V., Poljak, L., Luisi, B.F., and Carpousis, A.J. 2008. The RNase E of *Escherichia coli* is a membrane-binding protein. *Mol Microbiol*. 70:799-813.

Kime, L., Jourdan, S.S., Stead, J.A., Hidalgo-Sastre, A., and McDowall, K.J. 2010. Rapid cleavage of RNA by RNase E in the absence of 5' monophosphate stimulation. *Mol Microbiol*. 76:590-604.

Koslover, D.J., Callaghan, A.J., Marcaida, M.J., Garman, E.F., Martick, M., Scott, W.G., and Luisi, B.F. 2008. The crystal structure of the *Escherichia coli* RNase E apoprotein and a mechanism for RNA degradation. *Structure*. 16:1238-1244.

Lasa, I., Toledo-Arana, A., and Gingeras, T.R. 2012. An effort to make sense of antisense transcription in bacteria. *RNA Biol*. 9.

Le Derout, J., Régnier, P., and Hajnsdorf, E. 2002. Both temperature and medium composition regulate RNase E processing efficiency of the *rpsO* mRNA coding for ribosomal protein S15 of *Escherichia coli*. *J Mol Biol*. 319:341-349.

Lee, K., Zhan, X., Gao, J., Qiu, J., Feng, Y., Meganathan, R., Cohen, S.N., and Georgiou, G. 2003. RraA. a protein inhibitor of RNase E activity that globally modulates RNA abundance in *E. coli*. *Cell*. 114:623-634.

Lehnik-Habrink, M., Lewis, R.J., Mäder, U., and Stülke, J. 2012. RNA degradation in *Bacillus subtilis*: an interplay of essential endo- and exoribonucleases. *Mol Microbiol*. 84:1005-1017.

Lehnik-Habrink, M., Newman, J., Rothe, F.M., Solovyova, A.S., Rodrigues, C., Herzberg, C., Commichau, F.M., Lewis, R.J., and Stülke, J. 2011a. RNase Y in Bacillus subtilis: a Natively disordered protein that is the functional equivalent of RNase E from Escherichia coli. *J Bacteriol*. 193:5431-5441.

Lehnik-Habrink, M., Schaffer, M., Mader, U., Diethmaier, C., Herzberg, C., and Stülke, J. 2011b. RNA processing in Bacillus subtilis: identification of targets of the essential RNase Y. *Mol Microbiol*. 81:1459-1473.

Li, Z., Reimers, S., Pandit, S., and Deutscher, M.P. 2002. RNA quality control: degradation of defective transfer RNA. *EMBO J*. 21:1132-1138.

Liang, W., and Deutscher, M.P. 2010. A novel mechanism for ribonuclease regulation: transfer-messenger RNA (tmRNA) and its associated protein SmpB regulate the stability of RNase R. *J Biol Chem*. 285:29054-29058.

Liang, W., and Deutscher, M.P. 2012a. Post-translational modification of RNase R is regulated by stress-dependent reduction in the acetylating enzyme Pka (YfiQ). *RNA*. 18:37-41.

Liang, W., and Deutscher, M.P. 2012b. tmRNA-SmpB regulates RNase R turnover by promoting binding of HslUV and Lon proteases. *J Biol Chem*.

Liang, W., Malhotra, A., and Deutscher, M.P. 2011. Acetylation regulates the stability of a bacterial protein: growth stage-dependent modification of RNase R. *Mol Cell*. 44:160-166.

Lin-Chao, S., and Cohen, S.N. 1991. The rate of processing and degradation of antisense RNAI regulates the replication of ColE1-type plasmids *in vivo*. *Cell*. 65:1233-1242.

Link, T.M., Valentin-Hansen, P., and Brennan, R.G. 2009. Structure of *Escherichia coli* Hfq bound to polyriboadenylate RNA. *Proc Natl Acad Sci U S A*. 106:19292-19297.

Liou, G.G., Chang, H.Y., Lin, C.S., and Lin-Chao, S. 2002. DEAD box RhlB RNA helicase physically associates with exoribonuclease PNPase to degrade double-stranded RNA independent of the degradosome-assembling region of RNase E. *J Biol Chem*. 277:41157-41162.

Liou, G.G., Jane, W.N., Cohen, S.N., Lin, N.S., and Lin-Chao, S. 2001. RNA degradosomes exist *in vivo* in *Escherichia coli* as multicomponent

complexes associated with the cytoplasmic membrane via the N-terminal region of ribonuclease E. *Proc Natl Acad Sci U S A*. 98:63-68.

Lisitsky, I., and Schuster, G. 1999. Preferential degradation of polyadenylated and polyuridinylated RNAs by the bacterial exoribonuclease polynucleotide phosphorylase. *Eur J Biochem*. 261:468-474.

Lupold, D.S., Caoile, A.G., and Stern, D.B. 1999. Polyadenylation occurs at multiple sites in maize mitochondrial *cox2* mRNA and is independent of editing status. *Plant Cell*. 11:1565-1578.

Lybecker, M.C., Abel, C.A., Feig, A.L., and Samuels, D.S. 2010. Identification and function of the RNA chaperone Hfq in the Lyme disease spirochete Borrelia burgdorferi. *Mol Microbiol*. 78:622-635.

Mackie, G.A. 1992. Secondary structure of the mRNA for ribosomal protein S20. Implications for cleavage by ribonuclease E. *J Biol Chem*. 267:1054-1061.

Mäder, U., Zig, L., Kretschmer, J., Homuth, G., and Putzer, H. 2008. mRNA processing by RNases J1 and J2 affects *Bacillus subtilis* gene expression on a global scale. *Mol Microbiol*. 70:183-196.

Madhugiri, R., and Evguenieva-Hackenberg, E. 2009. RNase J is involved in the 5'-end maturation of 16S rRNA and 23S rRNA in *Sinorhizobium meliloti*. *FEBS Lett*. 583:2339-2342.

Majdalani, N., Cunning, C., Sledjeski, D., Elliott, T., and Gottesman, S. 1998. DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription. *Proc Natl Acad Sci U S A*. 95:12462-12467.

Majdalani, N., Hernandez, D., and Gottesman, S. 2002. Regulation and mode of action of the second small RNA activator of RpoS translation, RprA. *Mol Microbiol*. 46:813-826.

Marujo, P.E., Hajnsdorf, E., Le Derout, J., Andrade, R., Arraiano, C.M., and Régnier, P. 2000. RNase II removes the oligo(A) tails that destabilize the *rpsO* mRNA of *Escherichia coli*. *Rna*. 6:1185-1193.

Massé, E., Escorcia, F.E., and Gottesman, S. 2003a. Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. *Genes Dev*. 17:2374-2383.

Massé, E., Majdalani, N., and Gottesman, S. 2003b. Regulatory roles for small RNAs in bacteria. *Curr Opin Microbiol*. 6:120-124.

Mathy, N., Bénard, L., Pellegrini, O., Daou, R., Wen, T., and Condon, C. 2007. 5'-to-3' exoribonuclease activity in bacteria: role of RNase J1 in rRNA maturation and 5' stability of mRNA. *Cell*. 129:681-692.

Mathy, N., Hébert, A., Mervelet, P., Bénard, L., Dorléans, A., de la Sierra-Gallay, I.L., Noirot, P., Putzer, H., and Condon, C. 2010. *Bacillus subtilis*

ribonucleases J1 and J2 form a complex with altered enzyme behaviour. *Mol Microbiol*. 75:489-498.

Matos, R.G., Barbas, A., and Arraiano, C.M. 2009. RNase R mutants elucidate the catalysis of structured RNA: RNA-binding domains select the RNAs targeted for degradation. *Biochem J*. 423:291-301.

Matos, R.G., Barbas, A., Gomez-Puertas, P., and Arraiano, C.M. 2011. Swapping the domains of exoribonucleases RNase II and RNase R: conferring upon RNase II the ability to degrade ds RNA. *Proteins*. 79:1853-1867.

Matus-Ortega, M.E., Regonesi, M.E., Piña-Escobedo, A., Tortora, P., Dehò, G., and García-Mena, J. 2007. The KH and S1 domains of *Escherichia coli* polynucleotide phosphorylase are necessary for autoregulation and growth at low temperature. *Biochim Biophys Acta*. 1769:194-203.

McDowall, K.J., Hernandez, R.G., Lin-Chao, S., and Cohen, S.N. 1993. The *ams-1* and *rne-3071* temperature-sensitive mutations in the ams gene are in close proximity to each other and cause substitutions within a domain that resembles a product of the *Escherichia coli mre* locus. *J Bacteriol*. 175:4245-4249.

McDowall, K.J., Lin-Chao, S., and Cohen, S.N. 1994. A+U content rather than a particular nucleotide order determines the specificity of RNase E cleavage. *J Biol Chem*. 269:10790-10796.

Mikulecky, P.J., Kaw, M.K., Brescia, C.C., Takach, J.C., Sledjeski, D.D., and Feig, A.L. 2004. *Escherichia coli* Hfq has distinct interaction surfaces for DsrA, *rpoS* and poly(A) RNAs. *Nat Struct Mol Biol*. 11:1206-1214.

Mohanty, B.K., and Kushner, S.R. 1999. Analysis of the function of *Escherichia coli* poly(A) polymerase I in RNA metabolism. *Mol Microbiol*. 34:1094-1108.

Mohanty, B.K., and Kushner, S.R. 2000. Polynucleotide phosphorylase functions both as a 3' right-arrow 5' exonuclease and a poly(A) polymerase in *Escherichia coli*. *Proc Natl Acad Sci U S A*. 97:11966-11971.

Mohanty, B.K., and Kushner, S.R. 2002. Polyadenylation of *Escherichia coli* transcripts plays an integral role in regulating intracellular levels of polynucleotide phosphorylase and RNase E. *Mol Microbiol*. 45:1315-1324.

Mohanty, B.K., and Kushner, S.R. 2003. Genomic analysis in *Escherichia coli* demonstrates differential roles for polynucleotide phosphorylase and RNase II in mRNA abundance and decay. *Mol Microbiol*. 50:645-658.

Mohanty, B.K., and Kushner, S.R. 2006. The majority of *Escherichia coli* mRNAs undergo post-transcriptional modification in exponentially growing cells. *Nucleic Acids Res*. 34:5695-5704.

Mohanty, B.K., and Kushner, S.R. 2011. Bacterial/archaeal/organellar polyadenylation. *Wiley Interdiscip Rev RNA*. 2:256-276.

Mohanty, B.K., Maples, V.F., and Kushner, S.R. 2004. The Sm-like protein Hfq regulates polyadenylation dependent mRNA decay in *Escherichia coli*. *Mol Microbiol*. 54:905-920.

Molin, C., Jauhiainen, A., Warringer, J., Nerman, O., and Sunnerhagen, P. 2009. mRNA stability changes precede changes in steady-state mRNA amounts during hyperosmotic stress. *RNA*. 15:600-614.

Moll, I., Leitsch, D., Steinhauser, T., and Bläsi, U. 2003. RNA chaperone activity of the Sm-like Hfq protein. *EMBO Rep*. 4:284-289.

Montero Llopis, P., Jackson, A.F., Sliusarenko, O., Surovtsev, I., Heinritz, J., Emonet, T., and Jacobs-Wagner, C. 2010. Spatial organization of the flow of genetic information in bacteria. *Nature*. 466:77-81.

Morita, T., Maki, K., and Aiba, H. 2005. RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs. *Genes Dev*. 19:2176-2186.

Murashko, O.N., Kaberdin, V.R., and Lin-Chao, S. 2012. Membrane binding of *Escherichia coli* RNase E catalytic domain stabilizes protein structure and increases RNA substrate affinity. *Proc Natl Acad Sci U S A*. 109:7019-7024.

Nevo-Dinur, K., Nussbaum-Shochat, A., Ben-Yehuda, S., and Amster-Choder, O. 2011. Translation-independent localization of mRNA in *E. coli*. *Science*. 331:1081-1084.

Newman, J.A., Hewitt, L., Rodrigues, C., Solovyova, A., Harwood, C.R., and Lewis, R.J. 2011. Unusual, dual endo- and exonuclease activity in the degradosome explained by crystal structure analysis of RNase J1. *Structure*. 19:1241-1251.

Nielsen, J.S., Lei, L.K., Ebersbach, T., Olsen, A.S., Klitgaard, J.K., Valentin-Hansen, P., and Kallipolitis, B.H. 2010. Defining a role for Hfq in Gram-positive bacteria: evidence for Hfq-dependent antisense regulation in *Listeria monocytogenes*. *Nucleic Acids Res*. 38:907-919.

Niyogi, S.K., and Datta, A.K. 1975. A novel oligoribonuclease of *Escherichia coli*. I. Isolation and properties. *J Biol Chem*. 250:7307-7312.

Nurmohamed, S., Vaidialingam, B., Callaghan, A.J., and Luisi, B.F. 2009. Crystal structure of *Escherichia coli* polynucleotide phosphorylase core bound to RNase E, RNA and manganese: implications for catalytic mechanism and RNA degradosome assembly. *J Mol Biol*. 389:17-33.

Nurmohamed, S., Vincent, H.A., Titman, C.M., Chandran, V., Pears, M.R., Du, D., Griffin, J.L., Callaghan, A.J., and Luisi, B.F. 2011. Polynucleotide phosphorylase activity may be modulated by metabolites in *Escherichia coli*. *J Biol Chem*. 286:14315-14323.

O'Hara, E.B., Chekanova, J.A., Ingle, C.A., Kushner, Z.R., Peters, E., and Kushner, S.R. 1995. Polyadenylylation helps regulate mRNA decay in *Escherichia coli*. *Proc Natl Acad Sci U S A*. 92:1807-1811.

Otaka, H., Ishikawa, H., Morita, T., and Aiba, H. 2011. PolyU tail of rho-independent terminator of bacterial small RNAs is essential for Hfq action. *Proc Natl Acad Sci U S A*. 108:13059-13064.

Papenfort, K., Bouvier, M., Mika, F., Sharma, C.M., and Vogel, J. 2010. Evidence for an autonomous 5' target recognition domain in an Hfq-associated small RNA. *Proc Natl Acad Sci U S A*. 107:20435-20440.

Papenfort, K., Pfeiffer, V., Mika, F., Lucchini, S., Hinton, J.C., and Vogel, J. 2006. SigmaE-dependent small RNAs of *Salmonella* respond to membrane stress by accelerating global *omp* mRNA decay. *Mol Microbiol*. 62:1674-1688.

Papenfort, K., and Vogel, J. 2009. Multiple target regulation by small noncoding RNAs rewires gene expression at the post-transcriptional level. *Res Microbiol*. 160:278-287.

Perrin, R., Lange, H., Grienenberger, J.M., and Gagliardi, D. 2004. AtmtPNPase is required for multiple aspects of the 18S rRNA metabolism in *Arabidopsis thaliana* mitochondria. *Nucleic Acids Res*. 32:5174-5182.

Pertzev, A.V., and Nicholson, A.W. 2006. Characterization of RNA sequence determinants and antideterminants of processing reactivity for a minimal substrate of *Escherichia coli* ribonuclease III. *Nucleic Acids Res*. 34:3708-3721.

Pfeiffer, V., Papenfort, K., Lucchini, S., Hinton, J.C., and Vogel, J. 2009. Coding sequence targeting by MicC RNA reveals bacterial mRNA silencing downstream of translational initiation. *Nat Struct Mol Biol*. 16:840-846.

Piazza, F., Zappone, M., Sana, M., Briani, F., and Dehò, G. 1996. Polynucleotide phosphorylase of *Escherichia coli* is required for the establishment of bacteriophage P4 immunity. *J Bacteriol*. 178:5513-5521.

Portier, C., Dondon, L., Grunberg-Manago, M., and Régnier, P. 1987. The first step in the functional inactivation of the *Escherichia coli* polynucleotide phosphorylase messenger is a ribonuclease III processing at the 5' end. *EMBO J*. 6:2165-2170.

Prévost, K., Desnoyers, G., Jacques, J.F., Lavoie, F., and Massé, E. 2011. Small RNA-induced mRNA degradation achieved through both translation block and activated cleavage. *Genes Dev*. 25:385-396.

Prud'homme-Genereux, A., Beran, R.K., Iost, I., Ramey, C.S., Mackie, G.A., and Simons, R.W. 2004. Physical and functional interactions among RNase E, polynucleotide phosphorylase and the cold-shock protein, CsdA: evidence for a 'cold shock degradosome'. *Mol Microbiol*. 54:1409-1421.

Pruijn, G.J. 2005. Doughnuts dealing with RNA. *Nat Struct Mol Biol*. 12:562-564.

Purusharth, R.I., Klein, F., Sulthana, S., Jager, S., Jagannadham, M.V., Evguenieva-Hackenberg, E., Ray, M.K., and Klug, G. 2005. Exoribonuclease R interacts with endoribonuclease E and an RNA helicase in the psychrotrophic bacterium *Pseudomonas syringae* Lz4W. *J Biol Chem*. 280:14572-14578.

Py, B., Higgins, C.F., Krisch, H.M., and Carpousis, A.J. 1996. A DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome. *Nature*. 381:169-172.

Ramos, C.G., Sousa, S.A., Grilo, A.M., Feliciano, J.R., and Leitão, J.H. 2011. The second RNA chaperone, Hfq2, is also required for survival under stress and full virulence of *Burkholderia cenocepacia* J2315. *J Bacteriol*. 193:1515-1526.

Rasmussen, A.A., Eriksen, M., Gilany, K., Udesen, C., Franch, T., Petersen, C., and Valentin-Hansen, P. 2005. Regulation of *ompA* mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. *Mol Microbiol*. 58:1421-1429.

Raynal, L.C., and Carpousis, A.J. 1999. Poly(A) polymerase I of *Escherichia coli*: characterization of the catalytic domain, an RNA binding site and regions for the interaction with proteins involved in mRNA degradation. *Mol Microbiol*. 32:765-775.

Redko, Y., Bechhofer, D.H., and Condon, C. 2008. Mini-III, an unusual member of the RNase III family of enzymes, catalyses 23S ribosomal RNA maturation in *B. subtilis*. *Mol Microbiol*. 68:1096-1106.

Redon, E., Loubière, P., and Cocaign-Bousquet, M. 2005. Role of mRNA stability during genome-wide adaptation of *Lactococcus lactis* to carbon starvation. *J Biol Chem*. 280:36380-36385.

Reichenbach, B., Maes, A., Kalamorz, F., Hajnsdorf, E., and Görke, B. 2008. The small RNA GlmY acts upstream of the sRNA GlmZ in the activation of *glmS* expression and is subject to regulation by polyadenylation in *Escherichia coli*. *Nucleic Acids Res*. 36:2570-2580.

Repoila, F., and Darfeuille, F. 2009. Small regulatory non-coding RNAs in bacteria: physiology and mechanistic aspects. *Biol Cell*. 101:117-131.

Richards, J., Liu, Q., Pellegrini, O., Celesnik, H., Yao, S., Bechhofer, D.H., Condon, C., and Belasco, J.G. 2011. An RNA pyrophosphohydrolase triggers 5'-exonucleolytic degradation of mRNA in *Bacillus subtilis*. *Mol Cell*. 43:940-949.

Richards, J., Sundermeier, T., Svetlanov, A., and Karzai, A.W. 2008. Quality control of bacterial mRNA decoding and decay. *Biochim Biophys Acta*. 1779:574-582.

Russell, J.B. 2007. The energy spilling reactions of bacteria and other organisms. *J Mol Microbiol Biotechnol*. 13:1-11.

Sauer, E., Schmidt, S., and Weichenrieder, O. 2012. Small RNA binding to the lateral surface of Hfq hexamers and structural rearrangements upon mRNA target recognition. *Proc Natl Acad Sci U S A*. 109:9396-9401.

Sauer, E., and Weichenrieder, O. 2011. Structural basis for RNA 3'-end recognition by Hfq. *Proc Natl Acad Sci U S A*. 108:13065-13070.

Selinger, D.W., Saxena, R.M., Cheung, K.J., Church, G.M., and Rosenow, C. 2003. Global RNA half-life analysis in *Escherichia coli* reveals positional patterns of transcript degradation. *Genome Res*. 13:216-223.

Shahbabian, K., Jamalli, A., Zig, L., and Putzer, H. 2009. RNase Y, a novel endoribonuclease, initiates riboswitch turnover in *Bacillus subtilis*. *Embo J*. 28:3523-3533.

Sharma, C.M., Darfeuille, F., Plantinga, T.H., and Vogel, J. 2007. A small RNA regulates multiple ABC transporter mRNAs by targeting C/A-rich elements inside and upstream of ribosome-binding sites. *Genes Dev*. 21:2804-2817.

Shi, Z., Yang, W.Z., Lin-Chao, S., Chak, K.F., and Yuan, H.S. 2008. Crystal structure of *Escherichia coli* PNPase: central channel residues are involved in processive RNA degradation. *RNA*. 14:2361-2371.

Shimoni, Y., Friedlander, G., Hetzroni, G., Niv, G., Altuvia, S., Biham, O., and Margalit, H. 2007. Regulation of gene expression by small non-coding RNAs: a quantitative view. *Mol Syst Biol*. 3:138.

Sittka, A., Lucchini, S., Papenfort, K., Sharma, C.M., Rolle, K., Binnewies, T.T., Hinton, J.C., and Vogel, J. 2008. Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq. *PLoS Genet*. 4:e1000163.

Sittka, A., Pfeiffer, V., Tedin, K., and Vogel, J. 2007. The RNA chaperone Hfq is essential for the virulence of *Salmonella typhimurium*. *Mol Microbiol*. 63:193-217.

Slomovic, S., and Schuster, G. 2011. Exonucleases and endonucleases involved in polyadenylation-assisted RNA decay. *Wiley Interdiscip Rev RNA*. 2:106-123.

Smith, A.M., Fuchs, R.T., Grundy, F.J., and Henkin, T.M. 2010. Riboswitch RNAs: regulation of gene expression by direct monitoring of a physiological signal. *RNA Biol*. 7:104-110.

Söderbom, F., Binnie, U., Masters, M., and Wagner, E.G. 1997. Regulation of plasmid R1 replication: PcnB and RNase E expedite the decay of the antisense RNA, CopA. *Mol Microbiol*. 26:493-504.

Soper, T., Mandin, P., Majdalani, N., Gottesman, S., and Woodson, S.A. 2010. Positive regulation by small RNAs and the role of Hfq. *Proc Natl Acad Sci U S A*. 107:9602-9607.

Spickler, C., and Mackie, G.A. 2000. Action of RNase II and polynucleotide phosphorylase against RNAs containing stem-loops of defined structure. *J Bacteriol*. 182:2422-2427.

Stead, M.B., Marshburn, S., Mohanty, B.K., Mitra, J., Pena Castillo, L., Ray, D., van Bakel, H., Hughes, T.R., and Kushner, S.R. 2011. Analysis of *Escherichia coli* RNase E and RNase III activity *in vivo* using tiling microarrays. *Nucleic Acids Res*. 39:3188-3203.

Stickney, L.M., Hankins, J.S., Miao, X., and Mackie, G.A. 2005. Function of the conserved S1 and KH domains in polynucleotide phosphorylase. *J Bacteriol*. 187:7214-7221.

Storz, G., Vogel, J., and Wassarman, K.M. 2011. Regulation by small RNAs in bacteria: expanding frontiers. *Mol Cell*. 43:880-891.

Stougaard, P., Molin, S., and Nordstrom, K. 1981. RNAs involved in copy-number control and incompatibility of plasmid R1. *Proc Natl Acad Sci U S A*. 78:6008-6012.

Symmons, M.F., Jones, G.H., and Luisi, B.F. 2000. A duplicated fold is the structural basis for polynucleotide phosphorylase catalytic activity, processivity, and regulation. *Structure*. 8:1215-1226.

Tsai, Y.C., Du, D., Domínguez-Malfavón, L., Dimastrogiovanni, D., Cross, J., Callaghan, A.J., García-Mena, J., and Luisi, B.F. 2012. Recognition of the 70S ribosome and polysome by the RNA degradosome in *Escherichia coli*. *Nucleic Acids Res*.

Tsui, H.C., Leung, H.C., and Winkler, M.E. 1994. Characterization of broadly pleiotropic phenotypes caused by an hfq insertion mutation in *Escherichia coli* K-12. *Mol Microbiol*. 13:35-49.

Tuckerman, J.R., Gonzalez, G., and Gilles-Gonzalez, M.A. 2011. Cyclic di-GMP activation of polynucleotide phosphorylase signal-dependent RNA processing. *J Mol Biol*. 407:633-639.

Udekwu, K.I. 2010. Transcriptional and post-transcriptional regulation of the *Escherichia coli luxS* mRNA; involvement of the sRNA MicA. *PLoS One*. 5:e13449.

Udekwu, K.I., Darfeuille, F., Vogel, J., Reimegard, J., Holmqvist, E., and Wagner, E.G. 2005. Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. *Genes Dev*. 19:2355-2366.

van der Oost, J., Jore, M.M., Westra, E.R., Lundgren, M., and Brouns, S.J. 2009. CRISPR-based adaptive and heritable immunity in prokaryotes. *Trends Biochem Sci*. 34:401-407.

Vecerek, B., Moll, I., and Bläsi, U. 2007. Control of Fur synthesis by the non-coding RNA RyhB and iron-responsive decoding. *EMBO J*. 26:965-975.

Viegas, S.C., and Arraiano, C.M. 2008. Regulating the regulators: How ribonucleases dictate the rules in the control of small non-coding RNAs. *RNA Biol*. 5:230-243.

Viegas, S.C., Mil-Homens, D., Fialho, A.M., and Arraiano, C.M. 2012. Endoribonuclease mutations impair *Salmonella typhimurium* virulence. *submited*.

Viegas, S.C., Pfeiffer, V., Sittka, A., Silva, I.J., Vogel, J., and Arraiano, C.M. 2007. Characterization of the role of ribonucleases in *Salmonella* small RNA decay. *Nucleic Acids Res*. 35:7651-7664.

Viegas, S.C., Silva, I.J., Saramago, M., Domingues, S., and Arraiano, C.M. 2010. Regulation of the small regulatory RNA MicA by ribonuclease III: a target-dependent pathway. *Nucleic Acids Res*.

Vincent, H.A., and Deutscher, M.P. 2006. Substrate recognition and catalysis by the exoribonuclease RNase R. *J Biol Chem*. 281:29769-29775.

Vincent, H.A., and Deutscher, M.P. 2009a. Insights into how RNase R degrades structured RNA: analysis of the nuclease domain. *J Mol Biol*. 387:570-583.

Vincent, H.A., and Deutscher, M.P. 2009b. The roles of individual domains of RNase R in substrate binding and exoribonuclease activity. The nuclease domain is sufficient for digestion of structured RNA. *J Biol Chem*. 284:486-494.

Vogel, J., Bartels, V., Tang, T.H., Churakov, G., Slagter-Jäger, J.G., Hüttenhofer, A., and Wagner, E.G. 2003. RNomics in *Escherichia coli* detects new sRNA species and indicates parallel transcriptional output in bacteria. *Nucleic Acids Res*. 31:6435-6443.

Wang, Y., Liu, C.L., Storey, J.D., Tibshirani, R.J., Herschlag, D., and Brown, P.O. 2002. Precision and functional specificity in mRNA decay. *Proc Natl Acad Sci U S A*. 99:5860-5865.

Wassarman, K.M. 2007a. 6S RNA: a regulator of transcription. *Mol Microbiol*. 65:1425-1431.

Wassarman, K.M. 2007b. 6S RNA: a small RNA regulator of transcription. *Curr Opin Microbiol*. 10:164-168.

Waters, L.S., and Storz, G. 2009. Regulatory RNAs in bacteria. *Cell*. 136:615-628.

Xu, F., Lin-Chao, S., and Cohen, S.N. 1993. The *Escherichia coli pcnB* gene promotes adenylylation of antisense RNAI of ColE1-type plasmids *in vivo* and degradation of RNAI decay intermediates. *Proc Natl Acad Sci U S A*. 90:6756-6760.

Zangrossi, S., Briani, F., Ghisotti, D., Regonesi, M.E., Tortora, P., and Dehò, G. 2000. Transcriptional and post-transcriptional control of polynucleotide phosphorylase during cold acclimation in *Escherichia coli*. *Mol Microbiol*. 36:1470-1480.

Zilhão, R., Caillet, J., Régnier, P., and Arraiano, C.M. 1995a. Precise physical mapping of the *Escherichia coli rnb* gene, encoding ribonuclease II. *Mol Gen Genet*. 248:242-246.

Zilhão, R., Cairrão, F., Régnier, P., and Arraiano, C.M. 1996a. PNPase modulates RNase II expression in *Escherichia coli*: implications for mRNA decay and cell metabolism. *Mol Microbiol*. 20:1033-1042.

Zilhão, R., Camelo, L., and Arraiano, C.M. 1993. DNA sequencing and expression of the gene *rnb* encoding *Escherichia coli* ribonuclease II. *Mol Microbiol*. 8:43-51.

Zilhão, R., Plumbridge, J., Hajnsdorf, E., Régnier, P., and Arraiano, C.M. 1996b. *Escherichia coli* RNase II: characterization of the promoters involved in the transcription of *rnb*. *Microbiology*. 142 (Pt 2):367-375.

Zilhão, R., Régnier, P., and Arraiano, C.M. 1995b. The role of endonucleases in the expression of ribonuclease II in *Escherichia coli*. *FEMS Microbiol Lett*. 130:237-244.

Zuo, Y., Vincent, H.A., Zhang, J., Wang, Y., Deutscher, M.P., and Malhotra, A. 2006. Structural basis for processivity and single-strand specificity of RNase II. *Mol Cell*. 24:149-156.

Chapter 2

REGULATION OF THE SMALL REGULATORY RNA MICA BY RIBONUCLEASE III: A TARGET-DEPENDENT PATHWAY

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The author of this Dissertation had a major contribution in the work described in this chapter, both in the planning of the experimental work and in the performance of the experiments.

ABSTRACT

MicA is a *trans*-encoded small non-coding RNA, which down-regulates porin-expression in stationary-phase. In this work, we focus on the role of endoribonucleases III and E on *S.* Typhimurium sRNA MicA regulation. RNase III is shown to regulate MicA in a target-coupled way, while RNase E is responsible for the control of free MicA levels in the cell. We purified both *Salmonella* enzymes and demonstrated that *in vitro* RNase III is only active over MicA when in complex with its targets (whether *ompA* or *lamB* mRNAs). *In vivo*, MicA is demonstrated to be cleaved by RNase III in a coupled way with *ompA* mRNA. On the other hand, RNase E is able to cleave unpaired MicA and does not show a marked dependence on its 5' phosphorylation state. The main conclusion of this work is the existence of two independent pathways for MicA turnover. Each pathway involves a distinct endoribonuclease, having a different role in the context of the fine-tuned regulation of porin levels. Cleavage of MicA by RNase III in a target-dependent fashion, with the concomitant decay of the mRNA target, strongly resembles the eukaryotic RNAi system, where RNase III-like enzymes play a pivotal role.

INTRODUCTION

Small non-coding RNAs (sRNAs) play very important roles in post-transcriptional control of gene expression. MicF was the first *trans*-encoded antisense sRNA described and was discovered a little more than a quarter-century ago as a regulator of the *E. coli ompF* mRNA (Mizuno *et al.*, 1984). Following the advent of systematic genome wide sRNA searches, the total number of known sRNAs in *E. coli* and the model pathogen *Salmonella enterica* serovar Typhimurium has grown to well over a hundred (Livny and Waldor, 2007).

An extensive network of *trans*-antisense sRNAs have been shown to down-regulate the expression of several OMPs. While in some cases the same sRNA regulates multiple *omp* mRNAs (Guillier and Gottesman, 2006; Papenfort *et al.*, 2006), in other cases the same *omp* mRNA is target of multiple sRNAs (Douchin *et al.*, 2006; Johansen *et al.*, 2006; Papenfort *et al.*, 2006). OMPs are embedded within the outer membrane, which together with the peptidoglycan layer and the inner membrane form the bacterial cell envelope, the first barrier of defence against external aggressions. Coordination in the expression of *omp* genes seems critical for proper envelope assembly, and accounts for the existence of so many sRNAs to regulate OMP mRNAs.

To survive in a changing environment, bacteria must constantly adjust the nature and abundance of surface components. Any condition that unbalance OMP levels activates the response of the transcription factor σ^E (Alba and Gross, 2004; Ruiz and Silhavy, 2005) that triggers transcription of a set of genes, which collectively help the bacterium to recover from the stress condition. MicA and RybB are two of the σ^E activated genes in stationary phase, whose role is to immediately limit OMP synthesis (Figueroa-Bossi *et al.*, 2006; Johansen *et al.*, 2006;

Papenfort *et al.*, 2006). We have analysed the expression of MicA sRNA in wild-type cells at different phases of growth in LB and minimal media. In this experiment, we also included two growth conditions known to induce the two major *Salmonella* virulence regions, i.e. the *Salmonella* Pathogenicity Islands 1 and 2. The virulence genes encoded by SPI-1 facilitate the entry of *Salmonella* into non-phagocytic cells. The genes of SPI-2 encode virulence factors for intra-macrophage survival and systemic disease. MicA became detectable at early stationary phase of growth in LB medium, and strongly accumulated when growth further slowed down (Figure 1A). Interestingly, MicA levels under SPI-1 and SPI-2 inducing conditions were comparable to those in stationary phase (Figure 1A).

Both MicA and RybB act in the same fashion: they inhibit protein synthesis by base pairing to the translation initiation region of their mRNA targets in an Hfq-dependent manner, followed by the subsequent degradation of the mRNA. Although sRNAs generally modulate translational initiation by interfering with 30S ribosome loading, alterations of target mRNA levels are also often observed (Vogel and Wagner, 2007; Waters and Storz, 2009). A few studies performed in *E. coli* suggest that RNase cleavage of target mRNAs may be directly coupled to the degradation of the sRNA that is regulating the process, with both RNAs being degraded upon sRNA action (Afonyushkin *et al.*, 2005; Massé *et al.*, 2003; Vogel *et al.*, 2004).

RNases can have a major impact on sRNAs regulatory pathways by performing a key role in the biogenesis and processing of sRNAs, as well as in controlling their cellular levels through regulation of their turnover (Andrade and Arraiano, 2008; Arraiano *et al.*, 2010; Massé *et al.*, 2003; Morita *et al.*, 2005; Viegas and Arraiano, 2008; Viegas *et al.*, 2007). In *E. coli*, and presumably in many other Gram-negative bacteria, including *Salmonella*, mRNA decay is normally initiated by an endonucleolytic cleavage mainly performed by RNase E (Carpousis *et al.*,

2009) and, sometimes by RNase III (Conrad and Rauhut, 2002), followed by exoribonucleolytic degradation (Andrade *et al.*, 2009; Arraiano *et al.*, 2010). In *E. coli*, both endoribonucleases have also been implicated in the decay of sRNAs, upon translational silencing (Kaberdin and Blasi, 2006).

We have previously reported specific contributions of several *Salmonella* ribonucleases on the turnover of different sRNAs, namely MicA sRNA (Viegas *et al.*, 2007). MicA turnover was seen to be significantly dependent on the degradosome complex and PNPase (Figure 1B). We have also seen that in the absence of the double stranded-specific RNase III this sRNA is extremely stable (Figure 1C). At that time we hypothesized that this regulation of MicA by RNase III could involve the interaction with its target, since MicA forms an extended RNA duplex when binding to the mRNA that is close to the length ideal for RNase III substrates.

In this work, we have cloned and purified for the first time *Salmonella* RNase III and RNase E and have demonstrated that both endoribonucleases are responsible for the control of MicA sRNA levels. The role of the double stranded-specific endoribonuclease III over MicA only occurs through a target-dependent pathway, whether *in vitro* or *in vivo*. By contrast, the single stranded-specific endoribonuclease E is able to efficiently degrade free MicA sRNA. A model is proposed to explain the cooperation of both enzymes in the cell in order to achieve the fine-tuned control of the post-transcriptional regulator MicA.

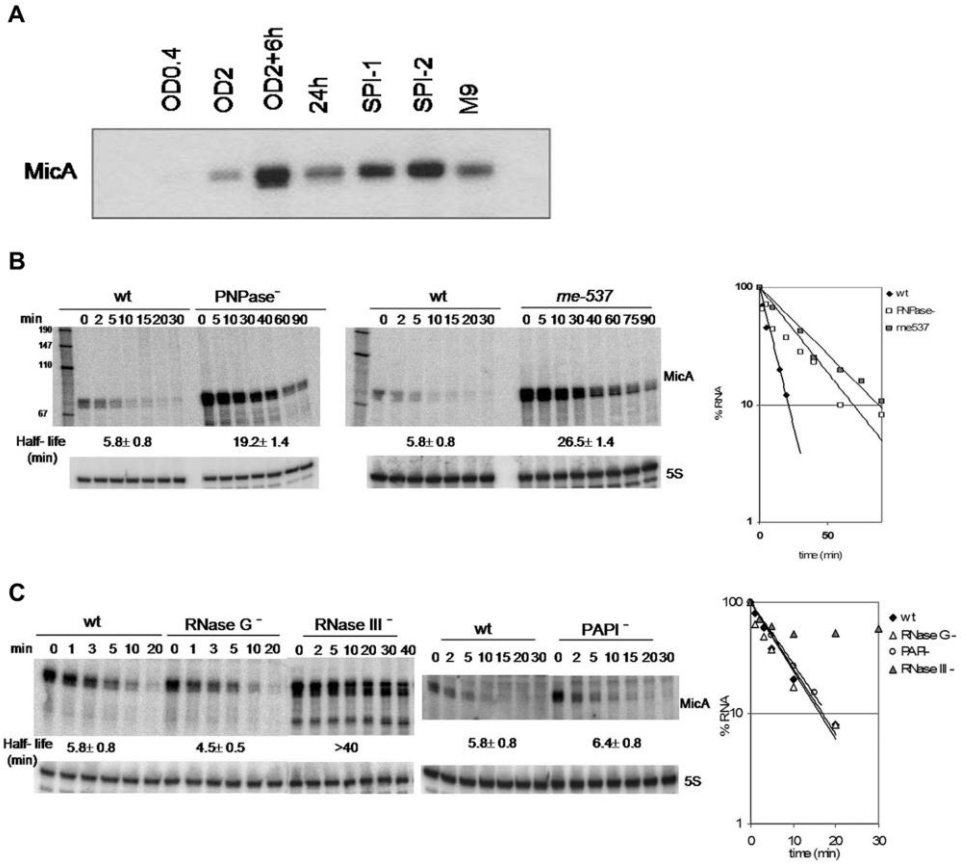


FIGURE 1 - Analysis of MicA turnover and expression under different growth conditions. (A) Northern blot analysis of sRNA expression in *Salmonella* SL1344 grown under different conditions as indicated in figure labels. **(B)** RNase E and PNPase mutations highly affect the stability of MicA sRNA. **(C)** Comparison of the effects of endoribonucleases G and III and PAP I in MicA stability. Adapted from (Viegas *et al.*, 2007).

EXPERIMENTAL PROCEDURES

Oligonucleotides

All oligonucleotides used in this study are listed in the Supplementary Table S1 and were synthesized by STAB Vida, Portugal.

Bacterial strains

All bacterial strains and plasmids used in this study are listed in the Tables 1 and 2, respectively. All *Salmonella* strains used are isogenic derivates of the wild-type *Salmonella enterica* serovar Typhimurium strain SL1344. The OmpA$^-$ (CMA-552), LamB$^-$ (CMA-554) and MicA$^-$ (CMA-555) mutants were constructed using the primer pairs pSV-104/pSV-105, pSV-108/pSV-109 and pSV-146/pSV-147, respectively, and following the λ-red recombinase method (Datsenko and Wanner, 2000), with few modifications, as previously described (Viegas *et al.*, 2007). All chromosomal mutations were subsequently transferred to a fresh SL1344 background by P22 HT105/1 int-201 transduction (Schmieger, 1971). The chloramphenicol-resistance cassette of plasmid pKD3 replaces nucleotides -190 to +1064 of the *ompA* gene, -20 to +1339 of *lamB* and +8 to +78 of *micA*. All gene deletions were verified by colony PCR using the primer pairs pSV-106/pSV-107 for *ompA*, pSV-110/pSV-111 for *lamB* and pSV-148/pSV-149 for *micA*. The *S.* Typhimurium RNase III deficient strain (CMA-551) was obtained by P22 transduction from SA5303 strain (Mattatall and Sanderson, 1998) and is tetracycline resistant. The double mutants were constructed using the same transduction method.

TABLE 1 - List of strains used in this work

Plasmid	Comments	Origin/Marker	Reference
pKD3	Template for mutants construction; carries chloramphenicol-resistance cassete	oriRγ/Amp^R	(Datsenko and Wanner, 2000)
pKD46	Temperature-sensitive λ-red recombinase expression plasmid	oriR101/Amp^R	(Datsenko and Wanner, 2000)
pCP20	Temperature-sensitive FLP recombinase expression plasmid	Amp^R, Cm^R	(Datsenko and Wanner, 2000)
pET-15b	Inducible expression vector, N-terminal His Tag	Amp^R	*Novagen*
pSVDA-01	pET-15b encoding His-RNase III	Amp^R	This study
pSVDA-02	pET-15b encoding His-RNase E	Amp^R	This study

TABLE 2 - List of plasmids used in this work

Strain	Relevant Markers / Genotype	Source/Reference
S. Typhimurium, SL1344	Str^R *hisG rpsL xyl*	(Hoiseth and Stocker, 1981)
CMA-537	SL1344 *rne-537* (Δ*rne*::Cm^R)	(Viegas *et al.*, 2007)
CMA-551	SL1344 *rnc-14*::ΔTn*10*, (Tc^R)	This study
CMA-552	SL1344 *ompA* (Δ*ompA*::Cm^R)	This study
CMA-554	SL1344 *lamB* (Δ*lamB*::Cm^R)	This study
CMA-555	SL1344 *micA* (Δ*micA*::Cm^R)	This study
CMA-556	SL1344 *rnc-14 micA* (*rnc-14*::ΔTn*10*/Δ*micA*::Cm^R)	This study
CMA-557	SL1344 *rnc-14 ompA* (*rnc-14*::ΔTn*10*/Δ*ompA*::Cm^R)	This study
CMA-558	SL1344 *rnc-14 rne-537* (*rnc-14*::ΔTn*10*/Δ*rne*::Cm^R)	This study
E. coli BL21(DE3)	F$^-$ *omp*T *hsd* S$_B$(*r*$_B^-$*m*$_B^-$) gal dcm (DE3)	(Studier and Moffatt, 1986)
E. coli BL21(DE3)*rec*A *rnc105*	F$^-$ *omp*T *hsd* S$_B$(*r*$_B^-$*m*$_B^-$) gal dcm (DE3) *rec*A *rnc*105	(Amarasinghe *et al.*, 2001)
E. coli DH5α	*rec*A1 *end*A1 *gyr*A96 thi-*hsd*R17 *sup*E44 *rel*A1 Δ*lac*ZYA-*arg* FU169 f80dLacZDM15	*New England Biolabs*

Bacterial growth

All strains were grown in LB broth at 37ºC with agitation throughout this study. SOC medium was used to recover transformants after heat shock (in the case of *E. coli*) or electroporation (in the case of *Salmonella*), before plating. Growth medium was supplemented with the following antibiotics when appropriate: ampicillin (150 μg/ml), chloramphenicol (25 μg/ml), streptomycin (90 μg/ml) and tetracycline (25 μg/ml).

RNA extraction and Northern blot analysis

Overnight cultures were diluted 1/100 in fresh LB medium and grown until 6 h after OD_{600} of 2 (OD2+6h). Culture samples were collected, mixed with 1 volume of stop solution [10 mM Tris (pH 7.2), 25 mM NaNO3, 5 mM $MgCl_2$, 500 μg/ml chloramphenicol] and harvested by centrifugation (10 min, 6000 g, 4ºC). For stability experiments, rifampicin (500 μg/ml) and nalidixic acid (20 μg/ml) were added to cells grown in LB at 37ºC, with agitation, till OD2+6. Incubation was continued and culture aliquots were withdrawn at the time-points indicated in the respective figures. RNA was isolated using the phenol/chlorophorm extraction method, precipitated in ethanol, resuspended in water and quantified on a Nanodrop 1000 machine (*Nanodrop Technologies*).

For northern blot analysis, 15 μg of total RNA was separated under denaturing conditions either by 8.3 M urea / 8% polyacrylamide gel in TBE buffer or by 1.3% agarose MOPS / formaldehyde gel. For polyacrylamide gels, transfer of RNA onto Hybond-N^+ membranes (*GE Healthcare*) was performed by electroblotting (1 h 50 min, 24 V, 4ºC) in TAE buffer. For agarose gels, RNA was transferred to Hybond-N^+ membranes by capillarity using 20% SSC as transfer buffer. In both cases, RNA was UV cross-linked to the membrane immediately after transfer. Membranes were then hybridized in RapidHyb Buffer (*GE*

Healthcare) at 68ºC for riboprobes and 43ºC in the case of oligoprobes and DNA probes. After hybridization, membranes were washed as described (Viegas *et al.*, 2007). Signals were visualized by PhosphorImaging (Storm Gel and Blot Imaging System, *Amersham Bioscience*) and analysed using the ImageQuant software (*Molecular Dynamics*).

Hybridization probes

Primers for templates amplification are listed in Supplementary Table S1. Labelling of the riboprobes and oligoprobes was performed as described (Viegas *et al.*, 2007). The riboprobes were obtained using the primer pair pSV-118/pSV-141 for MicA and pSV-142/pSV-143 for ompA. The DNA probe for 16S rRNA was generated using the primer pair pSV-144/pSV-145 and "Amersham MegaprimeTM DNA Labelling Systems" (*GE Healthcare*), according to the supplier instructions.

Construction of recombinant proteins

To overexpress *Salmonella* RNase E and RNase III proteins, the *rne* and *rnc* coding regions were amplified with primer pairs pSV-124/pSV-125 and pSV-129/pSV-130, respectively. The N-terminal region (comprising residues 1–522), corresponding to the catalytic domain of RNase E, was purified. In *E. coli*, the N-terminal half of RNase E (residues 1–498) was reported to be sufficient for the ribonuclease activity (McDowall and Cohen, 1996). The purified PCR products were double digested with BamHI and NdeI and ligated to the pET-15b vector previously digested with the same enzymes, yielding plasmids pSVDA-01 (*rnc*) and pSVDA-02 (*rne*). These plasmids were first cloned into *E. coli* DH5α and were subsequently transformed into BL21(DE3) strain in the case of pSVDA-02, and BL21(DE3) *rnc*105 *rec*A (Amarasinghe *et al.*, 2001) in the case of pSVDA-01 construction. This derivative strain of BL21(DE3), carrying an RNase III mutation,

was used because it blocks the auto-regulation of *Salmonella* RNase III by the endogenous *E. coli* homologue, resulting in a higher yield of the enzyme upon overexpression. All constructs were confirmed by DNA sequencing at STAB Vida.

Overexpression and purification of Salmonella RNase E and RNase III proteins

The BL21(DE3) strain and derivative, containing the recombinant plasmids of interest, were grown in 100 ml of LB medium supplemented with ampicillin (150 μg/ml) to an OD_{600} of 0.5. At this point, protein expression was induced by addition of 1 mM of IPTG for 3 h at 37ºC. Cells were then harvested by centrifugation and the pellets stored at -80ºC. The culture pellets expressing RNase III or RNase E were resuspended in 3 ml of Buffer A (20 mM Tris-HCl pH 8, 500 mM NaCl, 20 mM imidazole pH 8). Suspensions were lysed using a French Press at 900 psi in the presence of 0.1 mM of PMSF. After lysis, the crude extracts were treated with 125 U of Benzonase (*Sigma*) to degrade the nucleic acids and clarified by a 30 min centrifugation at 10000 g, 4ºC. The histidine tagged recombinant proteins were purified by affinity chromatography, using the ÄKTA FPLC™ System (*GE Healthcare*). The clarified extracts were loaded into a HisTrap HP Sepharose 1 ml column equilibrated in Buffer A. Protein elution was achieved in Buffer A with a linear imidazole gradient (from 20 to 500 mM). The fractions containing mostly the protein of interest, free of contaminants, were pooled. Eluted proteins were buffer exchanged with Desalting Buffer [10 mM Tris-HCl (pH 8), 3 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT] and concentrated by centrifugation at 4ºC with Amicon Ultra Centrifugal Filter Devices (*Millipore*), with a molecular mass cut-off of 10 kDa (RNase III) or 50 kDa (RNase E). Proteins were quantified using the Bradford Method (Bradford, 1976) and stored at -20ºC

in Desalting Buffer containing 50% (v/v) glycerol. The purity of the enzymes was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and revealed >90% homogeneity.

In vitro transcription and activity assays

DNA templates for the *in vitro* transcription were generated by PCR using chromosomal DNA from *S.* Typhimurium SL1344 strain. The phage T7 RNA polymerase promoter sequence was included in the forward primer sequences. *micA* was amplified with the primer pair pSV-116/pSV-117, *ompA* with pSV-122/pSV-123 and *lamB* with pSV-120/pSV-121. For the synthesis of the internally labelled 5′ triphosphate MicA, *in vitro* transcription was carried out using the purified PCR product as template in the presence of an excess of [^{32}P]-α –UTP over unlabelled UTP with 'Riboprobe *in vitro* Transcription System' (*Promega*) and T7 RNA polymerase. MicA substrate bearing 5′ monophosphate was obtained by adding an 8-fold excess of GMP over the other ribonucleotides to the *in vitro* transcription reaction. Non-radioactive molecules were transcribed in the same conditions but using equimolar concentrations of all four ribonucleotides. MicA transcripts were purified by electrophoresis on an 8.3 M urea / 10% polyacrylamide gel. The gel slice was crushed and the RNA eluted with elution buffer [3 M ammonium acetate pH 5.2, 1 mM EDTA, 2.5% (v/v) phenol pH 4.3], overnight at room temperature. The RNA was ethanol precipitated and resuspended in RNase free water. For the synthesis of the 5′-end-labelled MicA or *ompA*, *in vitro* transcription was carried out using the corresponding PCR product as template. MicA and *ompA* transcripts were run on a 10 or 6% polyacrylamide gel, respectively, identified by ethidium bromide (EtBr) staining and cut out from the gel. The RNA was eluted from the gel slice as described above. The RNA substrates were end-labelled with [^{32}P]-γ-ATP at 37ºC for 1 h, with 10 units of T4 polynucleotide kinase (*Fermentas*) using the supplier

exchange buffer and again purified from gel as above. The yield of the labelled substrates (cpm/µl) was determined by scintillation counting.

The hybridization between labelled and unlabelled substrates was always performed in a 1:40 molar ratio in the Tris component of the activity buffer by incubation for 10 min at 80ºC, followed by 45 min at 37ºC. The activity assays were done in a final volume of 50 µl containing the activity buffer {for RNase III [30 mM Tris-HCl pH 8, 160 mM NaCl and 0.1 mM DTT] and for RNase E [25 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 60 mM KCl, 100 mM NH_4Cl, 0.1 mM DTT and 5% (v/v) glycerol]} and ~10000 cpm of substrate. In the case of the activity assays with RNase III, 10 mM of $MgCl_2$ was added to the reaction mixture. As a control, prior to the beginning of each assay an aliquot was taken and was incubated until the end of the assay (without the enzyme). The reactions were started by the addition of the enzyme at a concentration of 500 nM, and further incubated at 37ºC in the case of RNase III and 30ºC for RNase E (Chelladurai *et al.*, 1991; Jiang *et al.*, 2000). Samples were withdrawn at the time-points indicated in the respective figures, and the reactions were stopped by the addition of formamide-containing dye supplemented with 10 mM EDTA. Reaction products were resolved in a 7 M urea / 15% or 8% polyacrylamide gel as indicated in the respective figure legends. Signals were visualized by PhosphorImaging and analyzed using ImageQuant software (*Molecular Dynamics*).

OMPs extraction and analysis

The membrane protein fraction from late stationary phase cultures (OD2+6h) was extracted as described (Matsuyama *et al.*, 1984). OMPs were analysed on 4% urea–SDS–12% polyacrylamide gel. Gels were stained overnight with Coomassie Brilliant Blue.

RESULTS

Detection of MicA sense transcripts in an RNase III- mutant

We have previously studied MicA sRNA turnover in *S.* Typhimurium and have analysed the particular contribution of several RNases to the decay of this sRNA.

We have found that the dsRNA-specific endoribonuclease III has a remarkable impact on the stability of MicA sRNA. In the wild-type, MicA sRNA has a half-life of ~6 min (Viegas *et al.*, 2007). In an RNase III- mutant, there was a dramatic stabilization of the sRNA (no significant decay in >2 h), with the concomitant accumulation of a degradation intermediate, very stable, which was absent in the wild-type (Viegas *et al.*, 2007) (see Figure 1C). In an RNase E mutant MicA was also stabilized, but the small stable intermediate was not detected (see Figure 1B).

We were interested in clarifying the nature of this small intermediate. For this purpose we have compared the bands pattern of isogenic RNase III+ and RNase III- strains, by northern blot analysis, with different probes. We have used a probe antisense (AS1) or sense (S1) to the 5'-end of MicA, which corresponds to the region of interaction with its targets (Bossi and Figueroa-Bossi, 2007; Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005). The same short MicA sRNA stable intermediate of ~45 nt (indicated by an asterisk in the figures) was detected with the antisense probe (AS1) only in the RNase III- strain (Figure 2A, left panel). When using the MicA sense probe (S1) we have also detected in this mutant a smaller transcript with approximately the same size (Figure 2A, right panel). Both species (sense and antisense) have a remarkably long half-life (Figure 2B). It was also detected with the sense probe another band with the size corresponding to that of MicA full transcript (74 nt). None of the bands observed with the sense probe were visible in the wild-type or the RNase E mutant (*rne-537*). Moreover,

the presence of these 'sense transcripts' is MicA dependent, since they were not detected in an RNase III$^-$ / MicA$^-$ strain.

The fact that the smaller transcript is equally present when using a sense or antisense probe and uniquely when RNase III is absent suggests that it is one strand of a stable dsRNA remnant of the MicA-target mRNA paired species. This smaller intermediate probably arises due to the previous activity of other degrading enzyme(s) but only accumulates in the absence of RNase III by virtue of its double stranded character. RNase E is probably a good candidate since the level of the smaller intermediate is decreased in an RNase III$^-$ mutant that is also impaired for degradosome formation - RNase III$^-$ / *rne-537* (Figure 2C). For instance, cleavage of MicA and *ompA* mRNA (a main target of MicA) by RNase E (Udekwu *et al.*, 2005) together with exoribonucleolytic degradation of both RNAs may explain why the antisense and sense transcripts have approximately the same size.

In order to confirm that the smaller transcripts correspond to a stable dsRNA remnant of the MicA-target mRNA paired species, we have used two other MicA sense probes differently located along the MicA transcript (Figure 2D). For each sense probe used, the correspondent antisense probe, complementary to MicA, was also designed. The location of each of the probes in the MicA sequence is indicated in the figure below the respective images. A transcript having the same size was detected whether with the sense probes S1 and S2, or with the corresponding AS1 and AS2 antisense probes. However, we have not obtained any signal when using a sense probe located in the 3'-end of MicA (S3), while the MicA full transcript could still be detected with the corresponding antisense probe (AS3).

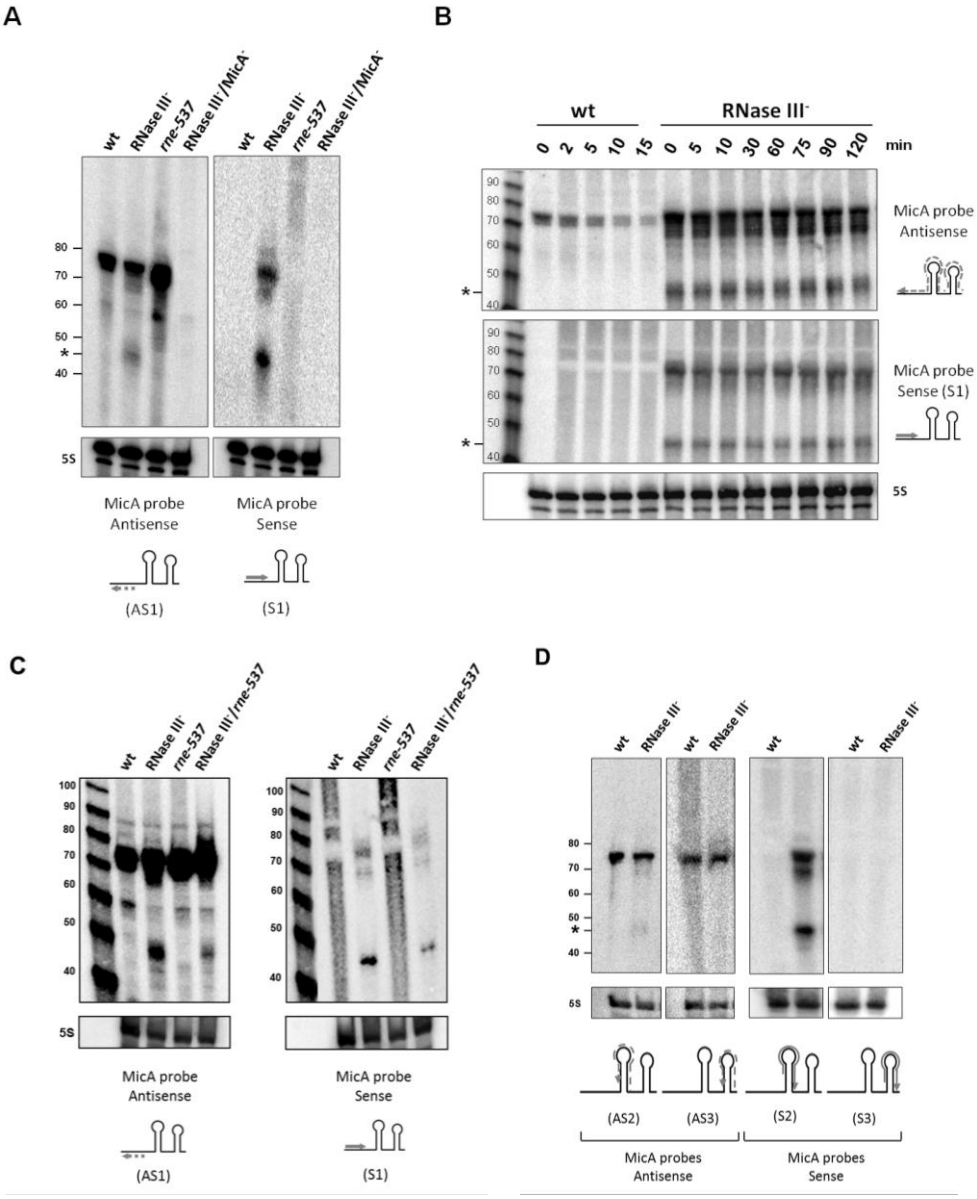


FIGURE 2 - Analysis of MicA sense and antisense species in single and double RNase III- and *rne-537* mutants. Total cellular RNA was extracted from the *S.* Typhimurium strains indicated and analyzed by northern blot. 15 μg of RNA (each lane) were separated on an 8% PAA / 8.3 M urea gel. The gel was then blotted to a Hybond-N+ membrane and hybridized with the corresponding probes. Details of RNA extraction and northern blot procedure are described in "Experimental Procedures" section. In each case, the membrane was stripped and then probed for 5S rRNA (pSV-139) as loading control. The radiolabelled marker 10 bp DNA Step Ladder (*Promega*) is on the left side. The respective sizes are represented in nucleotides (full MicA is 74 nt long). The asterisk indicates the fragment

that specifically accumulates in RNase III$^-$ strain. The probes used are indicated in the corresponding image. The arrow in each picture indicates the localization and direction of the probes in MicA sRNA: antisense (AS), represented by a dashed arrow; sense (S), represented by a solid arrow. The sequence of the probes is indicated in Supplementary Table S1. **(A)** Total RNA from *S.* Typhimurium wild-type and mutant derivatives RNase III$^-$, *rne-537* and RNase III$^-$/MicA$^-$ was hybridized with MicA antisense (AS1) and sense (S1) probes. The double mutant (RNase III$^-$/MicA$^-$) and the RNase E mutant (*rne-537*) were used as controls. **(B)** Comparison of the stability of both MicA sense and antisense species in the absence of RNase III. Total cellular RNA from wild-type and RNase III$^-$ mutant was extracted at the time-points (min) indicated on top, after transcription arrest. RNA samples were analyzed as described above using an antisense probe to the full MicA sequence (upper panel) or a sense probe (lower panel). **(C)** Total RNA from *S.* Typhimurium wild-type and mutant derivatives RNase III$^-$, *rne-537* and RNase III$^-$/*rne-537* was hybridized with MicA antisense (AS1) and sense (S1) probes. **(D)** Total RNA from wild-type and RNase III$^-$ mutant strains was hybridized with two other differently located antisense and sense probes, as indicated in the pictures below each image.

These results strongly indicate that the small degradation intermediate should correspond to a remnant of a duplex MicA-target mRNA. The lack of signal when using S3 (located in the 3'-end of MicA) further suggests that the duplex formation is confined to the 5'-end of MicA. This observation is in agreement with previous reports, which indicate that the interaction site is located in the 5'-end of the sRNA, at least for the two known targets of MicA (Bossi and Figueroa-Bossi, 2007; Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005).

MicA cleavage by RNase III is facilitated by base-pairing with its mRNA target(s)

The results presented in Figure 2 suggest that the cleavage of MicA by RNase III occurs in a target-dependent fashion. Taking this into account, together with the fact that RNase III is a double-stranded-specific endoribonuclease, led us to compare *in vitro* the activity of the enzyme both over MicA transcript alone or in complex with its mRNA targets. Until now only two targets for this sRNA have been described in *Salmonella*, *ompA* and *lamB* mRNAs. MicA was reported to act

over the translation initiation region of both molecules (Bossi and Figueroa-Bossi, 2007; Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005). In order to study the activity of *Salmonella* RNase III over MicA, we have cloned and purified the *Salmonella* enzyme as described in the "Experimental Procedures" section. An SDS-PAGE gel with the purified *Salmonella* RNase III is shown in Figure 3.

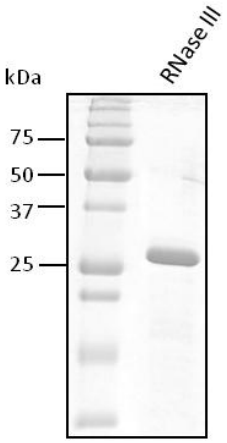


FIGURE 3 – SDS-PAGE analysis of the purified RNase III protein. Protein sample was visualized by Coomassie Brilliant Blue Staining. Molecular weight marker (Precision Plus Protein Pre-stained Standards – *Bio-Rad*) is shown on the left side of each image. Purified recombinant *Salmonella* RNase III (~27.5 kDa) was separated on a 15% polyacrylamide gel.

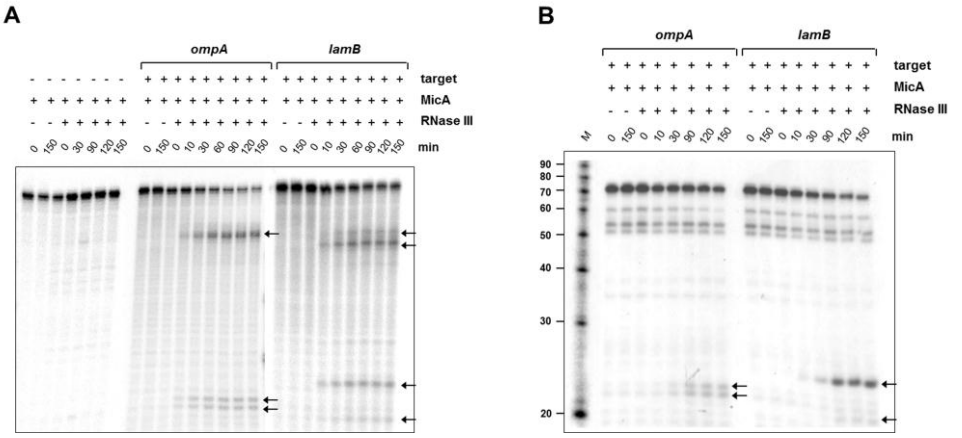
Activity assays were performed by incubating the purified *Salmonella* RNase III with α^{32}P-labelled MicA alone or in combination with the unlabelled 5'-UTR of *ompA* or *lamB* mRNAs. Since it has been shown that MicA is also able to bind *ompA* mRNA without the help of Hfq (Udekwu *et al.*, 2005), this protein was not included in the activity assays. MicA alone was found to be resistant to RNase III cleavage (Figure 4A). By contrast, in conditions favouring the hybridization of the sRNA transcript with each one of the target molecules, we could see the increasing accumulation of specific reaction products simultaneously with the disappearance of the substrate. This indicates that the formation of the sRNA-target mRNA complex promotes the RNase III cleavage of MicA.

The extension and location of MicA interaction with *ompA* or *lamB* mRNAs has been predicted to be slightly different (Bossi and Figueroa-Bossi, 2007; Udekwu *et al.*, 2005). Since RNase III cleaves dsRNA, the different interaction between MicA and the two targets could be in the origin of the distinct cleavage pattern induced by *ompA* or *lamB*. In order to identify the cleavage points generated by RNase III on the MicA-*ompA* and MicA-*lamB* hybrids, *in vitro* assays were performed as described above, but using 5'-end-labelled MicA in combination either with the unlabelled 5'-UTR of *ompA* or *lamB*. The results are shown in Figure 4B. RNase III cleavage generates two main fragments of 22 and 23 nt on MicA-*ompA* hybrid, and 21 and 25 nt on MicA-*lamB*. Since in this experiment MicA was 5'-end-labelled, the size of these fragments indicates the distance from the cleavage point to the 5'-end of MicA. The higher molecular weight bands observed only when MicA was internally labelled (Figure 4A) correspond to 3'-end fragments, since they are not detected in the cleavage of 5'-end-labelled MicA. A representation of the hybridization regions showing the RNase III cleavage positions in MicA sequence is presented in Figure 4C. All the cleavage positions are located inside the predicted region of interaction with each target, strongly supporting our hypothesis that RNase III is responsible for the coupled MicA-target degradation.

According to our proposal, cleavage of MicA is coupled with the mRNA target cleavage. In this sense the same kind of activity assays were carried out in order to check the direct activity of RNase III over the corresponding region of *ompA* mRNA. For this, the purified *Salmonella* RNase III was incubated with the 5'-end-labelled UTR of *ompA* (172 nt) alone or in combination with unlabelled MicA. As shown in Figure 4D although RNase III is able to cleave free *ompA*, a faster disappearance of the substrate when the hybrid *ompA*-MicA was used indicates that it is cleaved more efficiently. Moreover, the cleavage event gives

rise to specific degradation products that were not observed after incubation with *ompA* alone (Figure 4D). Among these products, we could observe the accumulation of fragments in the range of 113–130 nt, which is the expected size of fragments generated by cleavage inside the hybridization region with MicA (Figure 4C). The other products with a higher molecular weight probably arise due to alterations in the secondary structure of *ompA* after the duplex formation, which could generate a new dsRNA region suitable for RNase III. However, we cannot extrapolate to the *in vivo* situation, since these assays were performed with a truncated version of *ompA*. The ability of RNase III to preferentially cleave the hybrid *ompA*-MicA in the region corresponding to the hybridization between the two molecules is another evidence for the coupled degradation of the target and the sRNA.

Taken together, our results indicate that MicA decay *in vivo* is highly dependent on RNase III and its cleavage by this enzyme *in vitro* is triggered upon base-pairing with its target mRNAs.



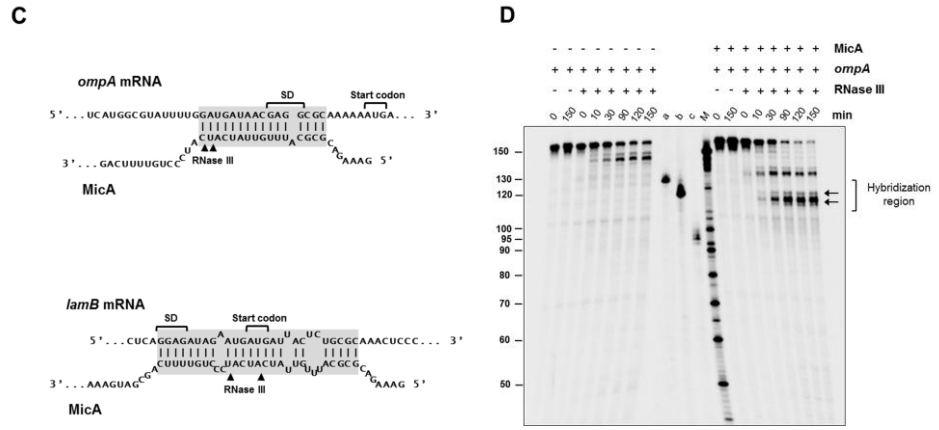


FIGURE 4 - *In vitro* cleavage of sRNA MicA or *ompA* 5′-UTR by RNase III. The radioactively labelled substrate was incubated with 500 nM of *Salmonella* RNase III. Aliquots withdrawn at the time-points indicated above each lane were analyzed on a 7 M urea / 15% or 8% PAA gel for MicA or *ompA*, respectively. The first two lanes of each reaction correspond to the controls without the protein at time zero (0) and at the end of the reaction time (150). The radiolabelled Decade Marker RNA (*Ambion*) is indicated by 'M'. The arrows in the figure indicate specific degradation products. **(A)** Assays performed with internally labelled MicA in the absence (-) (left panel) or in the presence (+) of a molar excess of *ompA* (middle panel) or *lamB* (right panel) unlabelled transcripts (5′-UTR sequence). **(B)** Assays performed with 5′-end-labeled MicA in the presence (+) of a molar excess of *ompA* (left panel) or *lamB* (right panel) unlabelled transcripts (5′-UTR sequence). The bands that are already observed in the absence of the enzyme (control reactions) arise due to the radiolysis of the substrate. **(C)** Proposed interaction regions of *ompA* and *lamB* mRNAs with MicA [adapted from (Bossi and Figueroa-Bossi, 2007)]. The Shine–Dalgarno regions of *ompA* and *lamB* are indicated. The arrows indicate the RNase III cleavage sites on MicA as determined on A and B. **(D)** Assays performed with 5′-end-labelled *ompA* in the absence (-) (on the left) or in the presence (+) (right panel) of a molar excess of unlabelled MicA. On the left side of the marker (M) radiolabelled transcripts of known sizes were included (a) 130 nt; (b) 120 nt and (c) 95 nt. The arrows in the figure indicate the degradation products located inside the hybridization region.

ompA expression is regulated by RNase III and is dependent on MicA

OmpA is a very abundant porin highly expressed in the exponential phase of growth. In stationary phase MicA is present at high levels and is the principal posttranscriptional down-regulator of the *ompA* mRNA (Rasmussen *et*

al., 2005; Udekwu *et al.*, 2005). Since our results indicate that MicA degradation by RNase III is target-dependent and we have observed the concomitant degradation of the *ompA* target mRNA *in vitro*, we analysed the effect of an RNase III$^-$ mutation on the levels of *ompA* mRNA in stationary phase. The RNase III$^-$ mutant shows an increment of almost 14-fold in *ompA* mRNA level in comparison to the wild-type (Figure 5A). A strong increase in the OmpA protein level was also observed. This suggests that the reduced levels of *ompA* mRNA observed in stationary phase in the wild-type (RNase III$^+$) are probably due to the cleavage and destabilization of the message by RNase III. This cleavage should be suppressed when RNase III is absent. This result strongly indicates that RNase III is implicated in the degradation of the *ompA* mRNA. On the other hand, the constant levels of OmpC and OmpD between the wild-type and RNase III$^-$, further suggest that RNase III is not involved in the turnover of their messages and that the control of these proteins levels in the cell follows a different pathway. Interestingly, Papenfort *et al.* (Papenfort *et al.*, 2006) have shown that MicA sRNA is also not involved in the control of *ompC* or *ompD* levels, while affecting *ompA*.

Since the degradation of both the sRNA MicA and the *ompA* target mRNA is dependent on RNase III, we have checked whether the RNase III regulation of *ompA* expression was also MicA dependent. Therefore, we have analysed the expression of *ompA* in a MicA$^-$ mutant strain. In stationary phase, MicA base-pairs with the 5'-UTR of *ompA* preventing ribosome binding and destabilizing the entire *ompA* mRNA (Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005). Accordingly, when the regulator is absent (MicA$^-$ mutant) the levels of *ompA* mRNA should be elevated. We observed an increase of about 7-fold in *ompA* mRNA levels when MicA is absent (Figure 5A). This result confirms that the control of *ompA* mRNA levels is dependent on MicA. However, the fact that in the absence of RNase III *ompA* mRNA levels are still higher than in the MicA$^-$

mutant indicates that RNase III may also have a role in *ompA* expression by an alternative pathway not involving MicA. In fact we show that RNase III is also able to cleave *ompA in vitro* in the absence of MicA. Additionally *ompA* mRNA may also be under the control of another sRNA in an RNase III dependent way.

If RNase III and MicA affect the *ompA* message through the same regulatory pathway, the combined absence of both would not result in a cumulative effect. In order to clarify this we have constructed and tested the effect of the double mutant RNase III$^-$/MicA$^-$ on the *ompA* mRNA levels. As shown in Figure 5A, in the double RNase III$^-$/MicA$^-$ mutant the *ompA* levels are reduced in comparison with the RNase III$^-$ single mutant, demonstrating that MicA and RNase III act over *ompA* message through a common pathway.

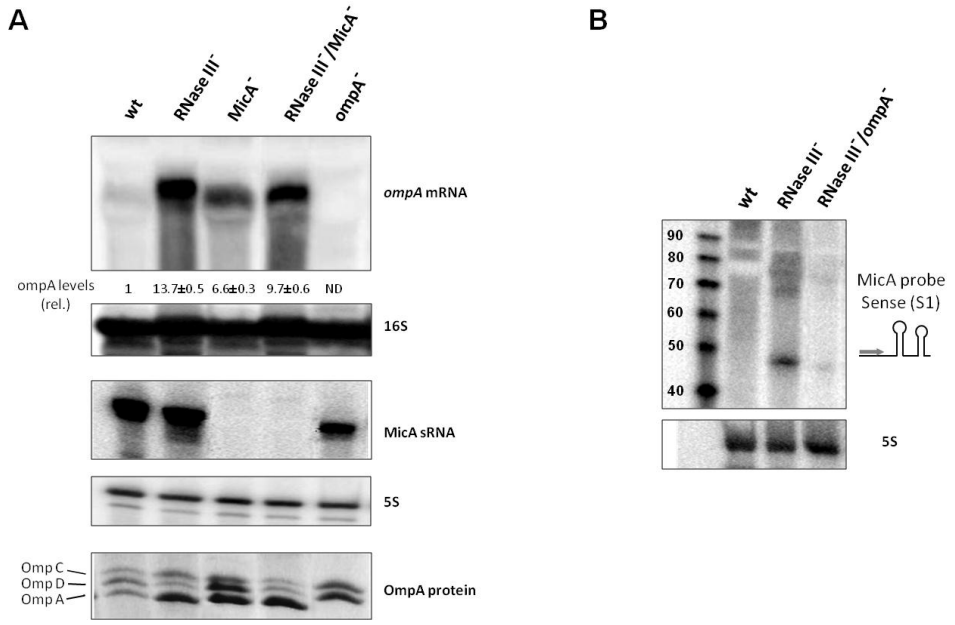


FIGURE 5 - Regulation of *ompA* and MicA expression in different mutant strains. Northern blot and SDS–PAGE analysis of RNA and protein samples extracted from wild-type and mutant strains as indicated on top of each lane. Details of experimental procedures are described in "Experimental Procedures" section. **(A)** (Upper panel) Analysis of steady-state *ompA* mRNA levels by northern blot. 15 µg of RNA (each lane) were resolved in a 1.3% formaldehyde-agarose gel. The gel was then blotted to a Hybond-

N^+ membrane and hybridized with the corresponding *ompA* riboprobe. Full-length transcripts were quantified using a Molecular Dynamics PhosphorImager. The amount of RNA found in wild-type was set as one. The ratio between each strain and the wild-type is depicted (relative levels). A representative membrane is shown and values indicated correspond to the average of several northern blot experiments with RNAs from at least two independent extractions. The membrane was stripped and then probed for 16S rRNA as loading control. (ND) Non-detectable. (Middle panel) MicA sRNA levels analysis by northern blot. 15 μg of RNA from the same mutants were separated on an 8% PAA / 8.3 M urea. The gel was then blotted to a Hybond-N^+ membrane and hybridized with the corresponding MicA riboprobe. The membrane was stripped and then probed for 5S rRNAs, as loading control. (Lower panel) Outer membrane protein fraction analysis by 4% urea–SDS–12% polyacrylamide gel electrophoresis. The positions of the OmpC, OmpD and OmpA bands are indicated. An $OmpA^-$ mutant was used as control. **(B)** Comparison of the levels of MicA sense species on the wild-type, RNase III^- and the double RNase III^-/$OmpA^-$ strains. The experimental procedure was similar to the one described in (A). The arrow on MicA sRNA picture indicates the localization and direction of the probe (S1). Loading control of the RNA was done with 5S rRNA probe and is represented below. Sizes were estimated using the radiolabelled 10 bp DNA Step Ladder (*Promega*), on the left side of the membrane.

Detection of the 'sense transcripts' in the RNase III^- mutant depends on ompA

Taken together, the results presented here point out that *ompA* is subjected to MicA-coupled degradation by RNase III. Since we had indications that the 'sense transcripts' detected in the RNase III^- mutant are remnants of the sRNA-target complex (see Figure 2), we have investigated if these 'sense-transcripts' corresponded to *ompA* mRNA fragments. Indeed, in the absence of both RNase III and *ompA*, the levels of these 'sense transcripts' are largely reduced when compared with those of the single RNase III^- mutant (Figure 5B). This means that the detection of these 'sense transcripts' is related with the presence of *ompA*, strongly suggesting that this mRNA might be one of the targets degraded by RNase III in conjunction with MicA.

The fact that the 'sense transcripts' are still detectable in the absence of *ompA* indicates that this mRNA might not be the only candidate for the MicA-coupled degradation. However, in a $LamB^-$ mutant (the other known target of

MicA) we did not observe, under our experimental conditions, a significant alteration in the level of the 'sense transcripts' (data not shown). Furthermore, in the absence of both *ompA* and *lamB* targets, the 'sense transcripts' could still be slightly observed (data not shown), indicating that other MicA targets subjected to the same type of regulation should exist in the cell.

RNase E cleaves 'free MicA' sRNA in vitro

We have demonstrated that the sRNA MicA degradation is influenced by RNase III. However, this seems to happen only in the presence of the target mRNA. As we have shown *in vitro*, the enzyme was not able to cleave MicA alone. Thus, the question of how is free MicA degraded remains to be answered. *In vivo* experiments have shown a large impact of an RNase E mutant on the levels and stability of full MicA sRNA (Viegas *et al.*, 2007). However, these results concern studies undertaken with the *rne-537* mutant derivative (Viegas *et al.*, 2007). Since this mutant only prevents degradosome formation, without totally abolishing the enzyme activity, we were also interested in clarifying the role of the catalytic activity of RNase E on the decay of MicA. Moreover, it has been shown that -A/U rich sequences together with adjacent stem-loop structures can comprise recognition sites for RNase E (Kaberdin *et al.*, 2000; Mackie, 1998). The sequence of the sRNA MicA matches these characteristics. Therefore, we have analyzed the ability of this endoribonuclease to cleave MicA sRNA transcript, *in vitro*. For this purpose we have cloned and purified the amino-terminal region of *Salmonella* RNase E. The homologous region in *E. coli* RNase E is known to be responsible for the catalytic activity of the enzyme (McDowall and Cohen, 1996). The results of the purification of the N-terminal segment of *Salmonella* RNase E are shown in Figure 6A.

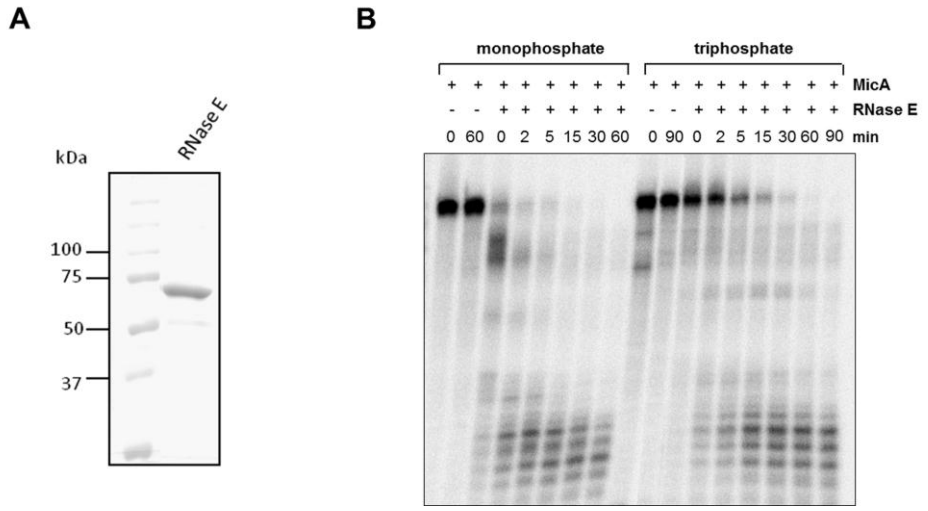


FIGURE 6 – *In vitro* study of MicA sRNA cleavage by RNase E. (A) SDS-PAGE analysis of the purified proteins. Protein sample was visualized by Coomassie Brilliant Blue Staining. Molecular weight marker (Precision Plus Protein Pre-stained Standards – *Bio-Rad*) is shown on the left side of each image. Purified N-terminal region of *Salmonella* RNase E (~64 kDa) was separated on a 10% polyacrylamide gel. **(B)** α-^{32}P-labelled MicA transcript, 5′ monophosphate (left panel) or 5′ triphosphate (right panel), was incubated with 500 nM of purified *Salmonella* RNase E (residues 1–522) at 30ºC. Aliquots withdrawn at the time-points indicated above each lane were analyzed on a 15% PAA / 7 M urea gel. The two first lanes of each reaction correspond to the controls without the protein both withdrawn at time zero and at the end of the reaction time.

In vitro assays with the purified protein were performed over uniformly labelled MicA transcript. It was seen before that RNase E preferentially cleaves RNAs with a 5′ monophosphate group over those endowed with a 5′ triphosphate (Celesnik *et al.*, 2007; Lin-Chao and Cohen, 1991; Mackie, 1998; Mackie, 2000). Thus, in the activity assays we have used as substrate both the monophosphate and the triphosphate MicA transcripts. Our results show that RNase E is able to cleave both substrates *in vitro* (Figure 6B), though the efficiency of cleavage was superior over monophosphorylated MicA. This is in agreement with the recent report that *E. coli* RNase E is also active over some triphosphate substrates (Kime *et al.*, 2010).

We have previously shown that in cells in which the degradosome scaffold of RNase E was deleted the degradation of MicA is slower (>4-fold stabilization) (Viegas *et al.*, 2007). This suggests that, *in vivo*, RNase E may need the cooperation of other degradosome components in the decay of this transcript. Indeed it was previously shown in *E. coli* and *Salmonella* that the absence of PNPase, the exoribonucleolytic component of the degradosome, has a remarkable impact over the stability of MicA sRNA (Andrade and Arraiano, 2008; Viegas *et al.*, 2007). However, the high ability of RNase E to cleave MicA *in vitro* indicates that the enzyme per se should importantly contribute for the *in vivo* degradation of free MicA.

DISCUSSION

Stress conditions that unbalance OMP levels activate the σ^E response, a complex set of changes normally devoted to protect the cell envelope from environmental challenges (Rowley *et al.*, 2006). The transcription factor σ^E triggers the synthesis of the sRNAs that control OMP levels (Johansen *et al.*, 2006; Papenfort *et al.*, 2006). Upon down-regulation of OMPs and the relief of membrane stress, the high sRNA levels have to be brought back to normal amounts. MicA sRNA is a σ^E-dependent porin down-regulator whose transcription is activated in stationary-phase (Figueroa-Bossi *et al.*, 2006; Johansen *et al.*, 2006; Papenfort *et al.*, 2006). Under this context, we were interested in studying the regulation of MicA cellular levels and determining the enzymes involved in this process.

MicA was previously found to be highly stabilized in cells lacking a functional RNase III (Viegas *et al.*, 2007). However, RNase III is not able to cleave MicA *in vitro*, suggesting that MicA alone is not a substrate for this enzyme.

Indeed, we demonstrate that RNase III is only able to cleave MicA *in vitro* when it base-pairs with its target(s). RNase III is a specific double-stranded RNA endoribonuclease, which plays multiple roles in the processing of rRNA and mRNA (Nicholson, 1999) and its activity has also been demonstrated over several sRNA-target complexes formed by *cis*-antisense sRNAs (Gerdes *et al.*, 1992; Jerome *et al.*, 1999; Krinke and Wulff, 1987; Simons and Kleckner, 1988). In these complexes, there is a perfect complementarity between the RNA partners, which constitutes a preferred substrate for RNase III, and avoids the need for Hfq. The limited complementary between *trans*-encoded sRNAs and their targets typically requires the help of the bacterial RNA chaperone Hfq. The *trans*-encoded sRNA MicA was shown to be dependent on Hfq both for stability and target degradation (Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005; Viegas *et al.*, 2007). It is generally assumed that Hfq binds both the regulator and the target RNA, favouring their interaction. Moreover, Hfq enhances the stability of many sRNAs *in vivo*, by protecting them from degradation (Storz *et al.*, 2005). Curiously, IstR-1 from *E. coli* and RNAIII from *S. aureus* are two *trans*-encoded sRNAs that act independently of Hfq and were also seen to be cleaved by RNase III in a target-coupled mechanism (Boisset *et al.*, 2007; Darfeuille *et al.*, 2007; Huntzinger *et al.*, 2005; Vogel *et al.*, 2004). Here we describe, in *Salmonella*, the first example of a system controlled by an Hfq-dependent *trans*-sRNA that involves the coupled degradation of the sRNA-target mRNA by RNase III.

In RNase III$^-$ cells, besides the high stabilization of MicA, a very stable smaller degradation intermediate is also observed. In agreement with the *in vitro* results, we have obtained several indications that this degradation intermediate corresponds to a remnant of a dsRNA complex formed by MicA and its target(s): (i) It is only detected in the RNase III$^-$ mutant (deficient for dsRNA degradation), where it is extremely stable by virtue of its double stranded character. When RNase III is present, dsRNA complexes are cleaved to products that are either

further degraded or too small to be detected by northern blot; (ii) This small intermediate is visible with both antisense MicA probes and sense probes complementary to the targets and (iii) The level detected with sense probes is highly reduced in the absence of OmpA (a main MicA target). Thus, this remnant species should indicate the region of interaction between MicA and its targets. Since a strong signal is detected with the 5′ probes and no signal at all is obtained with the probes located in the 3′-end, this region corresponds to the 5′ half of MicA. In fact, it is known that MicA interacts through its 5′-end sequence with its two targets described till now (Bossi and Figueroa-Bossi, 2007; Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005). Accordingly, the RNase III cleavage sites determined *in vitro* on both MicA-*ompA* and MicA-*lamB* hybrids are located in the 5′ half of MicA, inside the respective predicted hybridization region, which strongly correlates with the *in vivo* observations. This result is further confirmed by the fact that RNase III also cleaves the *ompA* 5′-UTR inside the same region, demonstrating that both molecules are cleaved together.

In the absence of both *ompA* and RNase III the level of the 'sense transcripts' is strongly decreased. The fact that these bands are still visible, despite at very low levels, may be related with the formation of complexes between MicA and other target(s), whose degradation should also be RNase III-dependent. We demonstrate that *in vitro* RNase III is also able to cleave the complex MicA-*lamB*. However, *in vivo*, whether in the absence of *lamB* or of both *lamB* and *ompA* we could still detect the sense transcripts referred above (data not shown). This means that probably besides *lamB* mRNA other *Salmonella* MicA targets (not yet identified) may exist in stationary-phase. In *E. coli*, expression of *phoPQ* has recently been shown to be repressed by MicA upon activation of σ^E (Coornaert *et al.*, 2010). In fact, MicA is a *trans*-encoded sRNA highly conserved in Enterobacteriaceae (Vogel and Papenfort, 2006). *Trans*-encoded sRNAs generally

establish short and imperfect interactions with its mRNA targets (~10–25 nt) (Kawamoto *et al.*, 2006; Waters and Storz, 2009), allowing the regulation of multiple targets by the same sRNA. For example, RybB sRNA controls more than 17 mRNAs, 10 of which encode OMPs, including *ompA*.

Upon MicA accumulation in stationary phase, the sRNA binds to *ompA* mRNA blocking ribosome binding and translation initiation. This releases the mRNA from the 'protection' by the ribosomes and leads to degradation of the ribosome-free mRNA by the concerted action of endo- and exoribonucleases (Arraiano *et al.*, 2010). In line with previous-work (Melefors and von Gabain, 1988; Vytvytska *et al.*, 1998), RNase E is thought to be the endoribonuclease responsible for the decay of *ompA* mRNA after the blockage of ribosome loading caused by MicA binding (Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005). In this report, we show that additionally the binding of MicA to *ompA* renders both RNAs susceptible to RNase III cleavage. We have demonstrated that this endoribonuclease is essential for *ompA* repression and, in agreement with previous studies (Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005), we observed a relieve of *ompA* repression in the absence of MicA. Both effects were seen to occur in a concerted way. The RNase III pathway has the advantage of simultaneously controlling the levels of the sRNA, whose function after the repression of the target will no longer be necessary in the cell. In addition RNase III cleavage makes the repression irreversible. From a physiological point of view, the existence of two distinct pathways may enhance the cell response in stress conditions allowing a fine-tuned balance of OmpA levels needed to keep the envelop integrity. Moreover by having two alternative degradation pathways the cell warrants the metabolism of molecules no longer needed. This may be crucial in stationary phase, which is characterized by limited resources.

Interestingly, the *ompA* levels in the double mutant (RNase III$^-$/MicA$^-$) are not restored to those observed in the MicA$^-$ mutant. This can be due to a direct

effect of RNase III over *ompA*. Indeed we show that *in vitro* RNase III is able to cleave *ompA* even in the absence of the sRNA. Alternatively, other(s) player(s) can be involved in the RNase III-mediated regulation of *ompA*. At least two other *trans*-encoded regulatory sRNAs (RseX and RybB) have been described as additional *ompA* regulators (Douchin *et al.*, 2006; Papenfort *et al.*, 2006).

We have just described a pathway for degradation of MicA sRNA that involves target binding and is dependent on RNase III. Our results show that RNase III cleaves MicA when hybridized with the targets. The question then arises how the levels of free MicA are brought back to normal when the cell no longer needs it. Our previous results *in vivo*, showed a high stabilization of MicA in an RNase E deletion mutant lacking the C-terminal region (Viegas *et al.*, 2007). This may suggest that RNase E needs the cooperation of other degradosome components in the degradation of MicA. In fact, we have previously shown that PNPase, the exonucleolytic component of the degradosome, has a great impact over the stability of this sRNA in *Salmonella*. According to the results presented here, the catalytic domain of RNase E also shows, *in vitro*, a high efficiency in the cleavage of this sRNA. This single stranded endoribonuclease seems to play an important role in the regulation of the abundance of free MicA.

For each sRNA the characterization of its turnover has to be analyzed from two different perspectives: the independent, and the dependent of target interaction. The later can be similar or not, whether the sRNA decay is influenced or not by the respective target(s). Taken together, the results presented in this study indicate the existence of two different pathways for MicA sRNA turnover, each one involving a specific endoribonuclease. According to the model proposed in Figure 7, when MicA is free, RNase E seems to take the control by efficiently degrading the sRNA. However, if MicA is interacting with the targets the target-dependent pathway of degradation predominates. This mechanism involves a

double stranded endoribonuclease that is able to degrade both the target and the sRNA, simultaneously.

Cleavage by RNase III within the sRNA–mRNA duplex and the subsequent decay of the mRNA intermediate by the cell machinery could rather resemble the RNAi scenario in eukaryotic organisms. RNase III-like enzymes are known to have a pivotal role in eukaryotic small noncoding RNA function and biogenesis (Arraiano *et al.*, 2010). Hence, it is not surprising that RNase III would also be a main player in the control of prokaryotic sRNA expression and function, broadening the enzyme's global role in the regulation of gene expression.

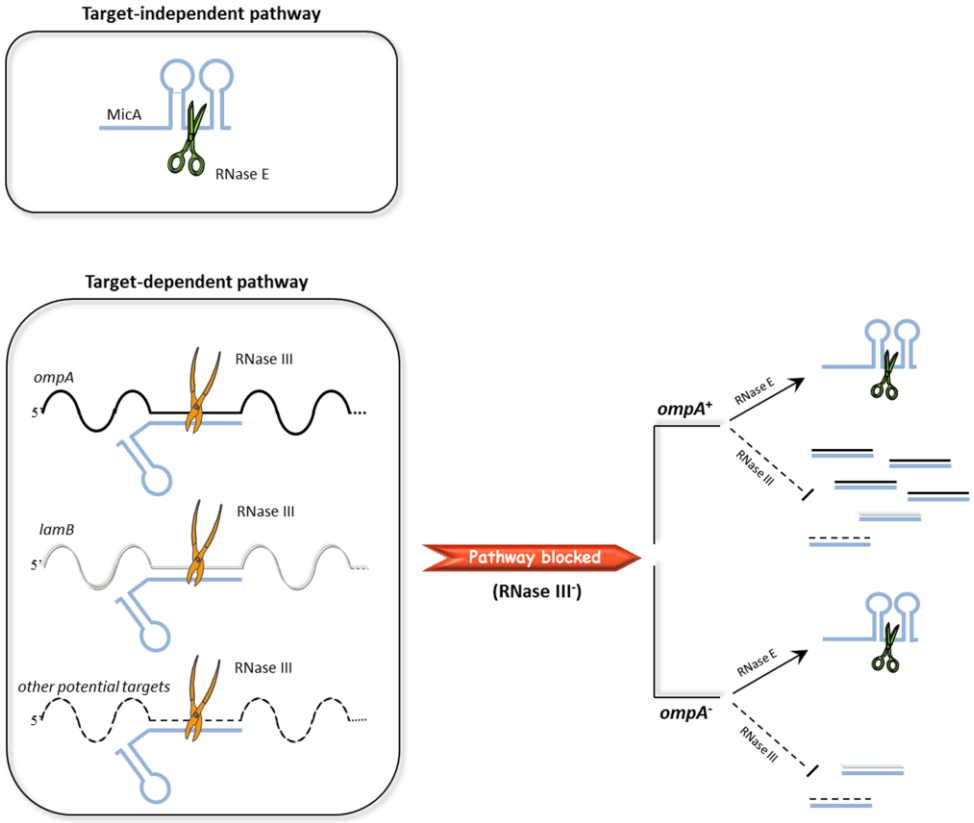


FIGURE 7 - Schematic representation of the two degradation pathways followed by MicA. RNase E and RNase III are represented by scissors and pliers, respectively. The two different pathways for MicA degradation are shown on the left side. The possible

associations of MicA with its targets are also depicted. In the wild-type, MicA and the targets should be fully degraded as a result of both degradation pathways in cooperation with the exoribonucleolytic activity. In the RNase III$^-$ mutant, the MicA-target dependent degradation by RNase III is blocked. As a result, some degradation intermediates are stabilized and can be detected, namely the target and MicA strands that have interacted but could not be cleaved by RNase III. *ompA* being the main target of MicA, its species are over-represented. When additionally the *ompA* target mRNA is absent, the respective degradation intermediate is no longer present in the cell and, as a consequence, there is a reduced level of transcripts detected with probes complementary to MicA-targets.

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REFERENCES

Afonyushkin, T., Vecerek, B., Moll, I., Blasi, U., and Kaberdin, V.R. 2005. Both RNase E and RNase III control the stability of *sodB* mRNA upon translational inhibition by the small regulatory RNA RyhB. *Nucleic Acids Res*. 33:1678-1689.

Alba, B.M., and Gross, C.A. 2004. Regulation of the *Escherichia coli* sigma-dependent envelope stress response. *Mol Microbiol*. 52:613-619.

Amarasinghe, A.K., Calin-Jageman, I., Harmouch, A., Sun, W., and Nicholson, A.W. 2001. *Escherichia coli* ribonuclease III: affinity purification of hexahistidine-tagged enzyme and assays for substrate binding and cleavage. *Methods Enzymol*. 342:143-158.

Andrade, J.M., and Arraiano, C.M. 2008. PNPase is a key player in the regulation of small RNAs that control the expression of outer membrane proteins. *RNA*. 14:543-551.

Andrade, J.M., Pobre, V., Silva, I.J., Domingues, S., and Arraiano, C.M. 2009. The role of 3'-5' exoribonucleases in RNA degradation. *Prog Mol Biol Transl Sci*. 85:187-229.

Arraiano, C.M., Andrade, J.M., Domingues, S., Guinote, I.B., Malecki, M., Matos, R.G., Moreira, R.N., Pobre, V., Reis, F.P., Saramago, M., Silva, I.J., and Viegas, S.C. 2010. The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol Rev*. 34:883-923.

Boisset, S., Geissmann, T., Huntzinger, E., Fechter, P., Bendridi, N., Possedko, M., Chevalier, C., Helfer, A.C., Benito, Y., Jacquier, A., Gaspin, C., Vandenesch, F., and Romby, P. 2007. *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. *Genes Dev*. 21:1353-1366.

Bossi, L., and Figueroa-Bossi, N. 2007. A small RNA downregulates LamB maltoporin in *Salmonella*. *Mol Microbiol*. 65:799-810.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 72:248-254.

Carpousis, A.J., Luisi, B.F., and McDowall, K.J. 2009. Endonucleolytic initiation of mRNA decay in *Escherichia coli*. *Prog Mol Biol Transl Sci*. 85:91-135.

Celesnik, H., Deana, A., and Belasco, J.G. 2007. Initiation of RNA decay in *Escherichia coli* by 5' pyrophosphate removal. *Mol Cell*. 27:79-90.

Chelladurai, B.S., Li, H., and Nicholson, A.W. 1991. A conserved sequence element in ribonuclease III processing signals is not required for accurate *in vitro* enzymatic cleavage. *Nucleic Acids Res*. 19:1759-1766.

Conrad, C., and Rauhut, R. 2002. Ribonuclease III: new sense from nuisance. *Int J Biochem Cell Biol*. 34:116-129.

Coornaert, A., Lu, A., Mandin, P., Springer, M., Gottesman, S., and Guillier, M. 2010. MicA sRNA links the PhoP regulon to cell envelope stress. *Mol Microbiol*. 76:467-479.

Darfeuille, F., Unoson, C., Vogel, J., and Wagner, E.G. 2007. An antisense RNA inhibits translation by competing with standby ribosomes. *Mol Cell*. 26:381-392.

Datsenko, K.A., and Wanner, B.L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A*. 97:6640-6645.

Douchin, V., Bohn, C., and Bouloc, P. 2006. Down-regulation of porins by a small RNA bypasses the essentiality of the regulated intramembrane proteolysis protease RseP in *Escherichia coli*. *J Biol Chem*. 281:12253-12259.

Figueroa-Bossi, N., Lemire, S., Maloriol, D., Balbontin, R., Casadesus, J., and Bossi, L. 2006. Loss of Hfq activates the sigmaE-dependent envelope stress response in *Salmonella enterica*. *Mol Microbiol*. 62:838-852.

Gerdes, K., Nielsen, A., Thorsted, P., and Wagner, E.G. 1992. Mechanism of killer gene activation. Antisense RNA-dependent RNase III cleavage ensures rapid turn-over of the stable *hok, srnB and pndA* effector messenger RNAs. *J Mol Biol*. 226:637-649.

Guillier, M., and Gottesman, S. 2006. Remodelling of the *Escherichia coli* outer membrane by two small regulatory RNAs. *Mol Microbiol*. 59:231-247.

Hoiseth, S.K., and Stocker, B.A. 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature*. 291:238-239.

Huntzinger, E., Boisset, S., Saveanu, C., Benito, Y., Geissmann, T., Namane, A., Lina, G., Etienne, J., Ehresmann, B., Ehresmann, C., Jacquier, A., Vandenesch, F., and Romby, P. 2005. *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate spa gene expression. *EMBO J*. 24:824-835.

Jerome, L.J., van Biesen, T., and Frost, L.S. 1999. Degradation of FinP antisense RNA from F-like plasmids: the RNA-binding protein, FinO, protects FinP from ribonuclease E. *J Mol Biol*. 285:1457-1473.

Jiang, X., Diwa, A., and Belasco, J.G. 2000. Regions of RNase E important for 5'-end-dependent RNA cleavage and autoregulated synthesis. *J Bacteriol*. 182:2468-2475.

Johansen, J., Rasmussen, A.A., Overgaard, M., and Valentin-Hansen, P. 2006. Conserved small non-coding RNAs that belong to the sigmaE regulon: role in down-regulation of outer membrane proteins. *J Mol Biol*. 364:1-8.

Kaberdin, V.R., and Blasi, U. 2006. Translation initiation and the fate of bacterial mRNAs. *FEMS Microbiol Rev*. 30:967-979.

Kaberdin, V.R., Walsh, A.P., Jakobsen, T., McDowall, K.J., and von Gabain, A. 2000. Enhanced cleavage of RNA mediated by an interaction between substrates and the arginine-rich domain of *E. coli* ribonuclease E. *J Mol Biol*. 301:257-264.

Kawamoto, H., Koide, Y., Morita, T., and Aiba, H. 2006. Base-pairing requirement for RNA silencing by a bacterial small RNA and acceleration of duplex formation by Hfq. *Mol Microbiol*. 61:1013-1022.

Kime, L., Jourdan, S.S., Stead, J.A., Hidalgo-Sastre, A., and McDowall, K.J. 2010. Rapid cleavage of RNA by RNase E in the absence of 5' monophosphate stimulation. *Mol Microbiol*. 76:590-604.

Krinke, L., and Wulff, D.L. 1987. OOP RNA, produced from multicopy plasmids, inhibits lambda *cII* gene expression through an RNase III-dependent mechanism. *Genes Dev*. 1:1005-1013.

Lin-Chao, S., and Cohen, S.N. 1991. The rate of processing and degradation of antisense RNAI regulates the replication of ColE1-type plasmids *in vivo*. *Cell*. 65:1233-1242.

Livny, J., and Waldor, M.K. 2007. Identification of small RNAs in diverse bacterial species. *Curr Opin Microbiol*. 10:96-101.

Mackie, G.A. 1998. Ribonuclease E is a 5'-end-dependent endonuclease. *Nature*. 395:720-723.

Mackie, G.A. 2000. Stabilization of circular *rpsT* mRNA demonstrates the 5'-end dependence of RNase E action *in vivo*. *J Biol Chem*. 275:25069-25072.

Massé, E., Escorcia, F.E., and Gottesman, S. 2003. Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. *Genes Dev*. 17:2374-2383.

Matsuyama, S., Inokuchi, K., and Mizushima, S. 1984. Promoter exchange between *ompF* and *ompC*, genes for osmoregulated major outer membrane proteins of *Escherichia coli* K-12. *J Bacteriol*. 158:1041-1047.

Mattatall, N.R., and Sanderson, K.E. 1998. RNase III deficient *Salmonella typhimurium* LT2 contains intervening sequences (IVSs) in its 23S rRNA. *FEMS Microbiol Lett*. 159:179-185.

McDowall, K.J., and Cohen, S.N. 1996. The N-terminal domain of the *rne* gene product has RNase E activity and is non-overlapping with the arginine-rich RNA-binding site. *J Mol Biol*. 255:349-355.

Melefors, O., and von Gabain, A. 1988. Site-specific endonucleolytic cleavages and the regulation of stability of *E. coli ompA* mRNA. *Cell*. 52:893-901.

Mizuno, T., Chou, M.Y., and Inouye, M. 1984. A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). *Proc Natl Acad Sci U S A*. 81:1966-1970.

Morita, T., Maki, K., and Aiba, H. 2005. RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs. *Genes Dev*. 19:2176-2186.

Nicholson, A.W. 1999. Function, mechanism and regulation of bacterial ribonucleases. *FEMS Microbiol Rev*. 23:371-390.

Papenfort, K., Pfeiffer, V., Mika, F., Lucchini, S., Hinton, J.C., and Vogel, J. 2006. SigmaE-dependent small RNAs of *Salmonella* respond to membrane stress by accelerating global *omp* mRNA decay. *Mol Microbiol*. 62:1674-1688.

Rasmussen, A.A., Eriksen, M., Gilany, K., Udesen, C., Franch, T., Petersen, C., and Valentin-Hansen, P. 2005. Regulation of *ompA* mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. *Mol Microbiol*. 58:1421-1429.

Rowley, G., Spector, M., Kormanec, J., and Roberts, M. 2006. Pushing the envelope: extracytoplasmic stress responses in bacterial pathogens. *Nat Rev Microbiol*. 4:383-394.

Ruiz, N., and Silhavy, T.J. 2005. Sensing external stress: watchdogs of the *Escherichia coli* cell envelope. *Curr Opin Microbiol*. 8:122-126.

Schmieger, H. 1971. The fate of the bacterial chromosome in P22-infected cells of *Salmonella typhimurium*. *Mol Gen Genet*. 110:238-244.

Simons, R.W., and Kleckner, N. 1988. Biological regulation by antisense RNA in prokaryotes. *Annu Rev Genet*. 22:567-600.

Storz, G., Altuvia, S., and Wassarman, K.M. 2005. An abundance of RNA regulators. *Annu Rev Biochem*. 74:199-217.

Studier, F.W., and Moffatt, B.A. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol*. 189:113-130.

Udekwu, K.I., Darfeuille, F., Vogel, J., Reimegard, J., Holmqvist, E., and Wagner, E.G. 2005. Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. *Genes Dev*. 19:2355-2366.

Viegas, S.C., and Arraiano, C.M. 2008. Regulating the regulators: How ribonucleases dictate the rules in the control of small non-coding RNAs. *RNA Biol*. 5:230-243.

Viegas, S.C., Pfeiffer, V., Sittka, A., Silva, I.J., Vogel, J., and Arraiano, C.M. 2007. Characterization of the role of ribonucleases in *Salmonella* small RNA decay. *Nucleic Acids Res*. 35:7651-7664.

Vogel, J., Argaman, L., Wagner, E.G., and Altuvia, S. 2004. The small RNA IstR inhibits synthesis of an SOS-induced toxic peptide. *Curr Biol*. 14:2271-2276.

Vogel, J., and Papenfort, K. 2006. Small non-coding RNAs and the bacterial outer membrane. *Curr Opin Microbiol*. 9:605-611.

Vogel, J., and Wagner, E.G. 2007. Target identification of small noncoding RNAs in bacteria. *Curr Opin Microbiol*. 10:262-270.

Vytvytska, O., Jakobsen, J.S., Balcunaite, G., Andersen, J.S., Baccarini, M., and von Gabain, A. 1998. Host factor I, Hfq, binds to *Escherichia coli ompA* mRNA in a growth rate-dependent fashion and regulates its stability. *Proc Natl Acad Sci U S A*. 95:14118-14123.

Waters, L.S., and Storz, G. 2009. Regulatory RNAs in bacteria. *Cell*. 136:615-628.

SUPPLEMENTARY INFORMATION

Supplementary Tables

SUPPLEMENTARY TABLE S1 - List of oligonucleotides used in this work. The restriction and T7 sequences in the primers used for cloning procedures and for riboprobe synthesis, respectively, are shown in bold and underlined.

Oligo	Sequence 5' to 3'*
pSV-104	CAGACGGAAACTTAAGCCTGCGGCTGAGTTACCACGTCTTTAACGCCTTTAACGATTGTGTAGGCTGGAGCTGCTTC
pSV-105	CGATTTATCATCTGAAACTGTTAAATGATGTGTATATCCGTCATGTTTTTTTCGGTCCATATGAATATCCTCCTTAG
pSV-106	TGCGAGAACGCTTGTCAGAA
pSV-107	ACAGGCGTTATTAGGCAAG
pSV-108	CGAAACGCAAAACCATTCGCAGTTTTAGAAGGTGGCAGCGTTTAAAGAAAAGGTGTAGGCTGGAGCTGCTTC
pSV-109	GCGCCCCTCGTTACGTCAGATGACCATCGTATTACCACCAGATTTCCATCGGTCCATATGAATATCCTCCTTAG
pSV-110	GTCGTCGTCTGCATCAAGAG
pSV-111	GCCTTATTCGGCTTACAAGC
pSV-146	AAATAAACTGAACTCTTTGTTCCGGGGCGAGTCTGAGTATATGAAAGACGTGTAGGCTGGAGCTGCTTC
pSV-147	GGCGGATACCGAGCCGTTTGCCGCGTGGCTTGCAAAACACGCCTGACCCAGGTCCATATGAATATCCTCCTTAG
pSV-148	AGAGCCGCTGGAGATTTTAC
pSV-149	TGGCATTTAGTCACCTCCG
pSV-116	GAAAT**TAATACGACTCACTATAGG**AAAGACGCGCATTTGTTAT
pSV-117	AAAAAGGCCACTCACGGAGTG
pSV-120	CCGTGGAAATCGACAGCCATTGCCTGAGCGGACATTAC
pSV-121	GGGCC**TAATACGACTCACTATAG**CTCAGGAGATAGAATGATGATTACTCTGC
pSV-122	GG**TAATACGACTCACTATAGG**CCAGGGGTGCTCGGCATAA
pSV-123	GCCAGTGCCACTGCAATCGCGATA
pSV-124	**GGAATTCCATATG**AAAAGAATGTTAATCAACGCG
pSV-125	**CGCGGATCC**CTACGTGGCGACGCTAACCG
pSV-126	TAATACGACTCACTATAGGG
pSV-127	TGCTAGTTATTGCTCAGCGG

Oligo	Sequence 5' to 3'*
pSV-128	GTGCGTACCGCGGGCGTGGG
pSV-129	**GGAATTCCATATG**AACCCCATCGTAATTAATC
pSV-130	**CGCGGATCC**TCATTCCAACTCCAGTTTTTTC
pSV-131	TAATACGACTCACTATAGGG
pSV-132	GTTAAATTGCTAACGCAGTCA
pSV-133	ACAAATGCGCGTCTTTC
pSV-134	TCATCGCTGAAAACAG
pSV-135	AGGCCACTCACGGAGTG
pSV-118	GAAAGACGCGCATTTGT
pSV-137	CCTGTTTTCAGCGAT
pSV-138	CACTCCGTGAGTGGCCT
pSV-139	CTACGGCGTTTCACTTCTGAGTTC
pSV-141	GTTTTTTTT**TAATACGACTCACTATAGG**GAGGCACGGAGTGGCCAAA
pSV-142	GGGCC**TAATACGACTCACTATAGG**GACCAGGTGTTATCTTTCGGAGCGGCCTGCGC
pSV-143	CAGCATAAGCCGTAGATATCGG
pSV-144	ACGGCTACCTTGTTACGACTT
pSV-145	AGAGTTTGATCCTGGCTCAG

Chapter 3

DISCOVERING THE ROLE OF THE SMALL RNA SRAL IN SALMONELLA TYPHIMURIUM

This chapter contains data from:

Silva, I.J., Viegas, S.C., Ortega, A.D., Portillo, F.G., and Arraiano, C.M. 2012. Discovering the role of the small RNA SraL in *Salmonella* Typhimurium. *In preparation*

ABSTRACT

In the last years, the study of a growing number of small non-coding RNAs in several prokaryotic organisms has unveiled unsuspected layers of regulation in a variety of cellular processes. In this chapter, we studied the role of SraL sRNA in *Salmonella* Typhimurium. This highly conserved sRNA was shown to be mostly expressed in late stationary phase of growth, the condition chosen for the study of its biological role. SraL appears to regulate some enzymes involved in carbohydrates metabolism, namely in the TCA cycle and oxidative phosphorylation pathways. Moreover, the expression of this sRNA was shown to be dependent on the glucose concentration of the growth medium. This analysis also suggested the regulation of additional targets involved in other processes. This regulation seems to occur by the base-pairing of the sRNA with the mRNA of its target(s). Thus, SraL seems to belong to the class of the *trans*-encoded sRNAs. This study represents the first attempt to unveil the role of SraL sRNA in *Salmonella* Typhimurium.

INTRODUCTION

Small non-coding RNAs have emerged as crucial components of the regulatory repertoires of both eukaryotic and prokaryotic organisms. These RNA molecules, which typically range from 50 to 250 nucleotides in length, constitute the most abundant class of post-transcriptional regulators in bacteria. They are often expressed under specific growth, stress or virulence conditions (Storz *et al.*, 2011). The most extensively studied sRNAs, often called antisense *trans*-encoded sRNAs, are those that regulate mRNAs by short and imperfect base-pairing interactions (Waters and Storz, 2009). The interaction between the sRNA and its target(s) may result in different effects. The most common effect described so far is the inhibition of translation of the targeted mRNA (Storz *et al.*, 2011). In this case, the sRNA binding to the mRNA occurs in the vicinity of the Shine-Dalgarno sequence, preceding the translational start codon. After the translational repression, the mRNA often becomes substrate for RNase E or RNase III (Caron *et al.*, 2010; Massé *et al.*, 2003).

The development of new RNomic techniques triggered the identification of a plethora of sRNAs in the last years (Sittka *et al.*, 2008; Sridhar *et al.*, 2010; Vogel *et al.*, 2003; Wassarman *et al.*, 2001; Zhang *et al.*, 2003). SraL (also known as RyjA) is a 140-nucleotides antisense sRNA firstly described in 2001 in two exhaustive genetic studies, in which a combination of different approaches was used in order to identify novel sRNAs in *E. coli* (Argaman *et al.*, 2001; Wassarman *et al.*, 2001). Subsequently, this sRNA was also detected in *Salmonella enterica* serovar Typhimurium (Ortega *et al.*, 2012; Viegas *et al.*, 2007). SraL sRNA is localized between the genes encoding *soxR* (Amábile-Cuevas and Demple, 1991) and a putative glutathione S-transferase (STM4267) but it is transcribed in the

opposite strand. Moreover, SraL seems to belong to the Hfq-dependent sRNAs group since in the absence of Hfq this sRNA was destabilized (Viegas *et al.*, 2007).

In this work, we have made the first attempts to investigate the biological role of SraL in *Salmonella*, since no targets were yet discovered for this sRNA. A proteomic analysis using *Salmonella* SraL mutant and SraL overexpressing strains detected the variation of the expression of several proteins involved in the metabolism of carbohydrates and also in protein folding. Interestingly, we also show that this sRNA is regulated by the levels of glucose in the cell. The relevance of the regulation of SraL over some of these putative targets is discussed in this chapter.

EXPERIMENTAL PROCEDURES

Oligonucleotides

All oligonucleotides used in this study are listed in the Table S1 in the "Supplementary Information" section, and were synthesized by STAB Vida.

Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are listed in the Table 1 and Table 2, respectively. All *Salmonella* strains used are isogenic derivates of the wild-type *Salmonella enterica* serovar Typhimurium strain SL1344. The *sraL* (CMA-651) null mutant was constructed using the primer pair pIS-001/pIS-002 and following the *λ-red* recombinase method (Datsenko and Wanner, 2000) with few modifications, as previously described (Viegas *et al.*, 2007). All chromosomal mutations were subsequently transferred to a fresh genetic background (SL1344 strain) by P22 HT105/1 int-201 transduction (Schmieger, 1971). The chloramphenicol-resistance cassette of plasmid pKD3 replaces nucleotides -9 to

+120 of the *sraL* gene. The gene deletion was verified by colony PCR using the primer pair pIS-003/pIS-004.

For construction of pISVA-01 plasmid expressing SraL, a PCR fragment containing the entire *sraL* sequence was amplified from SL1344 chromosome using the primer pair pIS-009/pIS-010. The resultant PCR fragment carrying a 5′-phosphate was cleaved with KpnI and ligated into the constitutive pZE12luc plasmid (blunt/KpnI site) (Lutz and Bujard, 1997). In this plasmid, the initiation site of the encoded RNA lies at position +1 of the constitutive PLlacO promoter of pZE12luc plasmid.

Competent *E. coli* DH5α cells (New English Biolabs) were used for cloning procedures during plasmid construction.

TABLE 1 - List of strains used in this work

Strain	Relevant Markers / Genotype	Source/Reference
S. Typhimurium, SL1344	Str^R *hisG rpsL xyl*	(Hoiseth and Stocker, 1981)
CMA-651	SL1344 *sraL* (Δ*sraL*::Cm^R)	This study
E. coli DH5α	*rec*A1 *end*A1 *gyr*A96 thi-*hsd*R17 *sup*E44 *rel*A1 Δ*lac*ZYA-*arg* FU169 f80dLacZDM15	*New England Biolabs*

TABLE 2 - List of plasmids used in this work

Plasmid	Comments	Origin/Marker	Reference
pKD3	Template for mutants construction; carries chloramphenicol-resistance cassete	oriRγ/Amp^R	(Datsenko and Wanner, 2000)
pKD46	Temperature-sensitive λ-red recombinase expression plasmid	oriR101/Amp^R	(Datsenko and Wanner, 2000)
pZE12Luc	P_{LlacO} promoter; constitutive expression plasmid	ColE1/Amp^R	(Lutz and Bujard, 1997)
pISVA-001	pZE12luc derivative; P_{LlacO} promoter; constitutive plasmid expressing SraL	ColE1/Amp^R	This study

Bacterial growth

All bacterial strains were grown in LB broth at 37ºC and 220 r.p.m. throughout this study. SOC medium was used to recover transformants after heat shock (in the case of *E. coli*) or electroporation (in the case of *Salmonella*), before plating. Conditions indicated as 'SPI-1 and SPI-2 inducing conditions' corresponded to growth in high salt (0.3 M NaCl) LB medium with low oxygen in sealed Falcon tubes, as described for SPI-1 induction (Sittka *et al.*, 2007), and in PCN minimal medium (1 mM phosphate buffer, pH 5.8) as described for SPI-2 induction (Lober *et al.*, 2006).

Growth medium was supplemented with the following antibiotics when appropriate: ampicillin (150 μg/ml), chloramphenicol (25 μg/ml) and streptomycin (90 μg/ml). For heat shock treatment, cells grown at 30ºC to an OD_{600} of 0.5 were transferred to 42ºC for 15 min. For cold shock treatment, cultures at an OD_{600} of 0.5 were transferred from 37ºC to 10ºC for 30 min and 4 h.

RNA extraction, Northern blot and Reverse Transcription-PCR (RT-PCR) analyses

Overnight cultures were diluted 1/100 in fresh medium and grown to the indicated cell densities at OD_{600} (growth medium and conditions are detailed in the respective figure legends). Culture samples were collected, mixed with 1 volume of stop solution (10 mM Tris pH 7.2, 25 mM $NaNO_3$, 5 mM $MgCl_2$, 500 μg/ml chloramphenicol), and harvested by centrifugation (10 min, 6000 g, 4ºC). RNA was isolated using the phenol/chlorophorm extraction method, precipitated in ethanol, resuspended in water and quantified on a Nanodrop 1000 machine (*NanoDrop Technologies*). The quality of the RNA was checked by agarose gel electrophoresis.

For Northern blot analysis, 15 μg of total RNA was separated under denaturing conditions either by 8.3 M urea / 6% polyacrylamide gel in TBE buffer

or by 1.3% agarose MOPS / formaldehyde gel. For polyacrylamide gels, transfer of RNA onto Hybond-N$^+$ membranes (*GE Healthcare*) was performed by electroblotting (1 h 50 min, 24 V, 4ºC) in TAE buffer. For agarose gels, RNA was transferred to Hybond-N$^+$ membranes by capillarity using 20x SSC as transfer buffer. In both cases, RNA was UV crosslinked to the membrane immediately after transfer. Membranes were then hybridized in PerfectHyb Buffer (*Sigma*) at 68ºC for riboprobes and 43ºC in the case of oligoprobes. After hybridization, membranes were washed as previously described (Viegas *et al.*, 2007). Signals were visualized by PhosphorImaging (Storm Gel and Blot Imaging System, *Amersham Bioscience*) and analyzed using the ImageQuant software (*Molecular Dynamics*).

RT-PCR reactions were performed using total RNA with the OneStep RT-PCR kit (*Quiagen*). Reactions were mainly carried out according to the supplier's instructions. Modifications were introduced regarding the amount of RNA and number of PCR cycles, depending on gene expression levels. The primer pairs used for the analysis of the different putative targets are indicated in the Supplementary Table S1 in the "Supplementary Information" section. As a control, 16S rRNA was amplified with specific primers pIS-018/pIS-019. Prior to RT-PCR, all RNA samples were treated with Turbo DNA free Kit (*Ambion*). Control experiments, run in the absence of reverse transcriptase, yielded no product.

Hybridization probes

Primers for templates amplification are listed in Supplementary Table S1 in the "Supplementary Information" section. The riboprobes were obtained using the primer pairs pIS-021/pIS-022, pIS-043/pIS-044, pIS-045/pIS-046, pIS-047/pIS-048, pIS-049/pIS-050, pIS-051/pIS-052 and pIS-017/pIS-054 for SraL, MicA, GlpD,

SfcA, RfbH, NuoG and Tig, respectively. 5S rRNA and 16S rRNA were detected by the 5'-end-labelled oligonucleotides pIS-023 and pIS-024, respectively. Riboprobes were generated from PCR fragments (a T7 RNA polymerase promoter sequence was added by the antisense primer) in the presence of an excess of [^{32}P]-α-UTP over unlabelled UTP using the T7 RNA polymerase from *Promega*. DNA oligonucleotides were labelled with [^{32}P]-γ-ATP using T4 polynucleotide kinase (*Fermentas*). All labelled probes were purified over G50 columns (*GE Healthcare*) to remove unincorporated nucleotides prior to hybridization.

Proteomic analysis

For the proteomic analysis, overnight cultures were diluted 1/100 in fresh LB medium and grown until 6h after OD_{600} of 2. Then, 2 OD units of each culture were transferred to a tube containing 0.25 volumes of stop solution (5% phenol / 95% ethanol) and kept on ice for 30 min. Cells were sedimented by centrifugation for 10 min at 3200 g 4ºC, washed with stop solution, centrifuged again and stored at -80ºC. Pellets were lysed in Laemmli sample buffer (1.3% SDS, 10%, v/v, glycerol, 50 mM Tris/HCl, 1.8% β-mercaptoethanol, pH 6.8) and the total protein estimated using the Bradford reagent (*Bio-Rad*). Approximately 30 μg of total protein (corresponding to approximately 0.3 ODs) were run in SDS-PAGE 12% gel. Loading equivalence among the samples was confirmed by checking GroEL by western blot analysis. Five slices from mid-run gels were submitted to in-gel tryptic digestion, and the tryptic peptide mixtures processed for protein identification by liquid chromatography-tandem mass spectrometry as described (García-del Portillo *et al*., 2011).

Sequence retrieval and alignments

BlastN was used for sequence retrieval (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) of the following genome sequences: *Salmonella enterica* serovar Typhimurium LT2 (NC_003197), *Salmonella enterica* serovar Typhi Ty2 (NC_004631), *Salmonella bongori* NCTC 12419 (NC_015761), *Shigella boydii* CDC 3083-94 (NC_010658), *Shigella flexneri* 2a str. 301 (NC_004337), *Shigella dysenteriae* Sd197 (NC_007606), *Escherichia coli K12* (NC_000913), *Citrobacter rodentium* ICC168 (NC_013716), *Citrobacter koseri* ATCC BAA-895 (NC_009792), *Enterobacter sp.* 638 (NC_009436), *Klebsiella pneumoniae* 342 (NC_011283). Alignments were made using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

RESULTS AND DISCUSSION

SraL sRNA is conserved among several enteric bacteria

SraL sRNA (also known as RyjA) was first discovered in two independent studies in *E. coli* in which the use of comparative genomics and microarrays allowed the identification of novel sRNAs (Argaman *et al.*, 2001; Wassarman *et al.*, 2001). More recently, it was confirmed that this sRNA is also expressed in *S.* Typhimurium (Ortega *et al.*, 2012; Viegas *et al.*, 2007).

Since SraL expression was detected in both *E. coli* and *S.* Typhimurium we performed an extensive search of SraL gene over the genomes of other enteric bacteria using BlastN tool. This sRNA was shown to be highly conserved, being identified in bacteria such as *Shigella*, *Citrobacter* and *Klebsiella* (Figure 1).

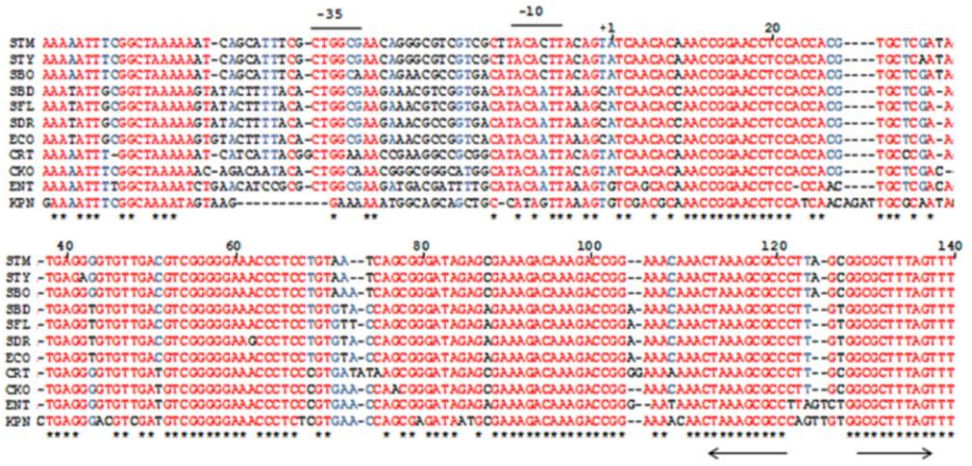

FIGURE 1 - Alignment of the *sraL* gene including the upstream promoter region in several enterobacteria. All nucleotides are coloured regarding their degree of conservation (red: high conservation; blue: partial conservation; black: little or no conservation). The asterisks (*) below the sequences are indicating the nucleotides conserved between all the species analysed. The putative -10 and -35 boxes of the *sraL* promoter are indicated (Argaman *et al.*, 2001). "+1" marks the transcriptional start site. The Rho (ρ)-independent terminator is indicated by arrows. STM: *Salmonella enterica* serovar Typhimurium; STY: *Salmonella enterica* serovar Typhi; SBO: *Salmonella bongori*; SBD: *Shigella boydii*; SFL: *Shigella flexneri*; SDR: *Shigella dysenteriae*; ECO: *Escherichia coli*; CRT: *Citrobacter rodentium*; CKO: *Citrobacter koseri*; ENT: *Enterobacter*; KPN: *Klebsiella pneumoniae.*

Using the *MFold* program (http://mfold.rna.albany.edu/?q=mfold) (Zuker, 2003) we predicted related secondary structures for SraL sRNA of several enteric bacteria. The most stable predicted structure of the sRNA in *S.* Typhimurium is shown in Figure 2A. Despite some small differences, the majority of SraL structures represented show a high resemblance and all contain the same *Rho*-independent terminator (Figure 2A-F). There are evidences that this *Rho*-independent terminator contributes also to the stabilization of the sRNAs (Abe and Aiba, 1996; Aiba *et al.*, 1991).

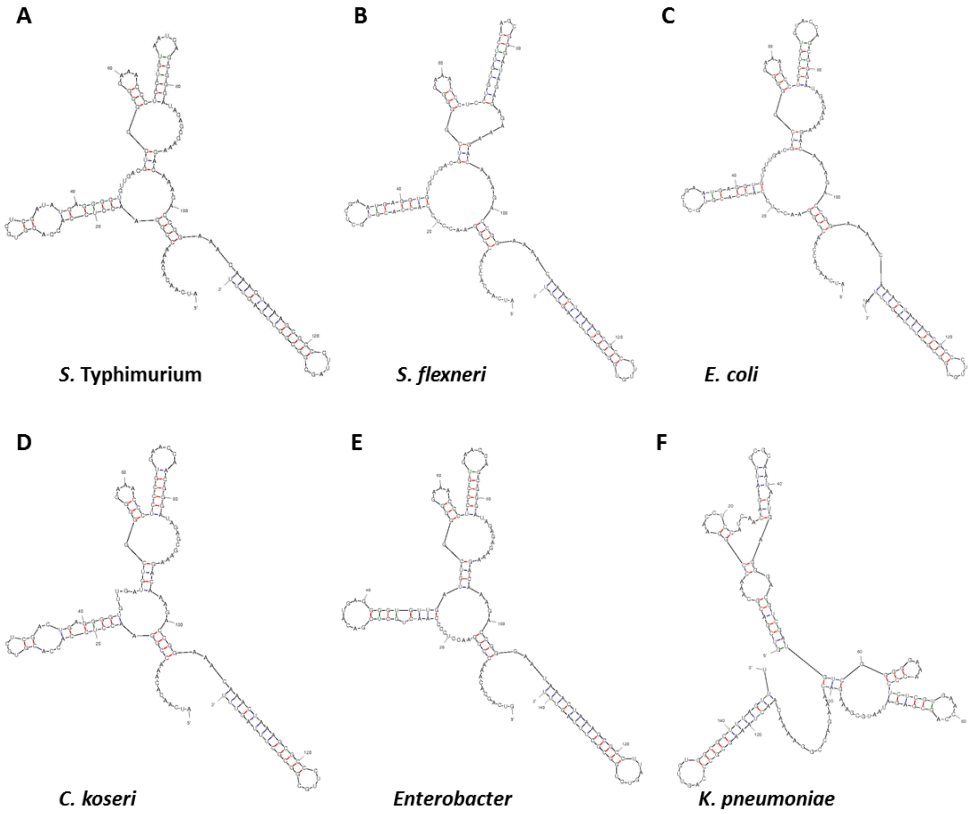

FIGURE 2 – SraL sRNA structures predicted by *Mfold* program (Zuker, 2003) of **(A)** *Salmonella* Typhimurium; **(B)** *Shigella flexneri*; **(C)** *Escherichia coli*; **(D)** *Citrobacter koseri*; **(E)** *Enterobacter*; **(F)** *Klebsiella pneumoniae*.

We have compared the expression of *S.* Typhimurium SraL under different growth conditions. When cells are grown in LB at 37ºC, SraL is mostly detected upon entry in stationary phase of growth (Figure 3). The highest expression of the sRNA was achieved in the growth for 6h after OD2. Moreover, this sRNA is also expressed in conditions that induce the expression of the genes of the *Salmonella* pathogenicity island 2, indicating a possible role of SraL in *Salmonella* virulence (Figure 3). More specifically, SraL seems to be necessary after internalization of this bacterium into host cells since its expression is much higher

under SPI-2 inducing conditions. In fact, it was recently shown that SraL is expressed in intracellular bacteria located inside fibroblasts at 24h post-infection (Ortega *et al.*, 2012). SraL is also expressed in cells subjected to heat shock, cells grown for 4h under cold-shock and also under SPI-1 inducing conditions, but at much lower levels (Figure 3).

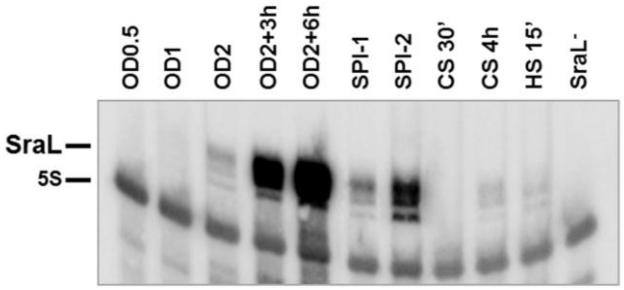

FIGURE 3 – Analysis of SraL sRNA expression under different growth conditions. Cells were grown in LB at 37ºC till OD_{600} of 0.5 (OD0.5), 1 (OD1), 2 (OD2), 3 h after OD2 (OD2+3h) and 6 h after OD2 (OD2+6h). Cells were also grown under conditions of induction of SPI-1 and SPI-2 genes. For heat and cold shock, cells were grown in LB till OD_{600} of 0.5 and then subjected to cold shock (10ºC) for 30 min and 4 h (CS 30' and CS 4h, respectively) and heat shock (42ºC) for 15 min (HS 15'). 15 µg of total RNA were run on a 6% PAA / 8.3 M urea gel. SraL was detected using a riboprobe; probing for 5S rRNA was used as a loading control.

Proteomic analysis of cells with different SraL expression levels

Although there are a few studies about SraL, the biological function of this sRNA was not yet revealed. To identify SraL targets we analyzed in parallel the proteome of *S.* Typhimurium wild-type, a *sraL* null mutant and a *sraL* overexpressing strain. Proteomic analysis had already been employed to identify several targets of other sRNAs, such as the well-known MicA sRNA that was seen to down-regulate *ompA* mRNA levels (Rasmussen *et al.*, 2005; Udekwu *et al.*, 2005) and Spot42 sRNA which overexpression specifically reduced the synthesis of GalK in *E. coli* (Moller *et al.*, 2002). We performed this analysis using cells in late stationary phase of growth, the condition in which this sRNA is mostly expressed (Figure 3) (Viegas *et al.*, 2007). For this analysis, we have first constructed a *sraL*

null mutant strain in which we deleted the entire sequence of the gene and also an overexpressing strain in which the SraL gene was cloned into a constitutive expression plasmid. We have observed that, under our growth conditions, these strains have no significant differences in growth in comparison with the wild-type strain (data not shown). By Northern blot analysis we could confirm the absence of SraL in the mutant strain and also its overexpression in the strain carrying the SraL overexpressing plasmid (Figure 4).

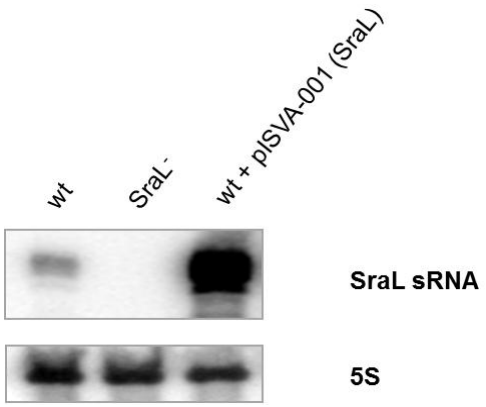

FIGURE 4 – Analysis of SraL sRNA expression. Total cellular RNA was extracted from the *S.* Typhimurium strains grown in LB at 37ºC till 6 h have passed after they reached OD_{600} of 2. 15 µg of RNA were separated on a 6% PAA / 8.3 M urea gel. The gel was then blotted to a Hybond-N^+ membrane and hybridized with the corresponding SraL riboprobe. Probing for 5S rRNA confirmed equal loading.

A total of 713 proteins were identified in our proteomic analysis and quantified across the three strains analyzed (see Supplementary Table S2 in "Supplementary Information" section). The majority of the proteins mostly affected by the change of SraL levels in the cell are those involved in the metabolism of carbohydrates (Table 3). Therefore, SraL sRNA seems to be implicated somehow in the carbohydrate metabolism, especially in the pathways that involve glucose. In fact, other sRNAs have been implicated in the control of making and breaking of sugars (Görke and Vogel, 2008).

TABLE 3 – List of the proteins mostly affected by the change of SraL levels in the cell

		Unique peptides		
Identified Proteins	**Acession #**	**wt[a]**	**ΔSraL[b]**	**pSraL[c]**
Aerobic sn-glycerol-3-phosphate dehydrogenase (GlpD)	STM3526	21	25	18
Trigger factor (Tig)	STM0447	14	20	11
Malate synthase A (AceB)	STM4183	10	17	7
NAD-linked malate dehydrogenase (SfcA)	STM1566	10	15	9
Putative fructose-1,6-biphosphate aldolase (YneB)	STM4078	8	5	9
Succinate-semialdehyde dehydrogenase I (GabD)	STM2791	7	5	11
CDP-6deoxy-D-xylo-4-hexulose-3-dehydrase (RfbH)	STM2090	8	10	5
NADH dehydrogenase I chain G (NuoG)	STM2323	7	10	4
Putative inner membrane protein (YhjG)	STM3610	4	8	1
Anaerobic sn-glycerol-3-phosphate dehydrogenase (GlpC)	STM2286	2	9	0

[a]wild-type SL1344 strain
[b] SraL deletion mutant
[c] SraL overexpressing strain

RT-PCR analysis of cells with and without SraL expression

Several *trans*-encoded sRNAs base-pair with their mRNA target(s) at the RBS, thus blocking translation by preventing ribosome binding. In most cases where ribosome binding is blocked, an associated decrease in the stability of the mRNA is also observed, possibly as an indirect result of the blocking of ribosome entry. Since the expression of several proteins was affected by the different levels of SraL in the cell we performed a screening in which we compared the mRNA expression levels of the putative targets between the wild-type and the SraL deletion mutant strain, using RT-PCR and Northern blot analyses (Figure 5).

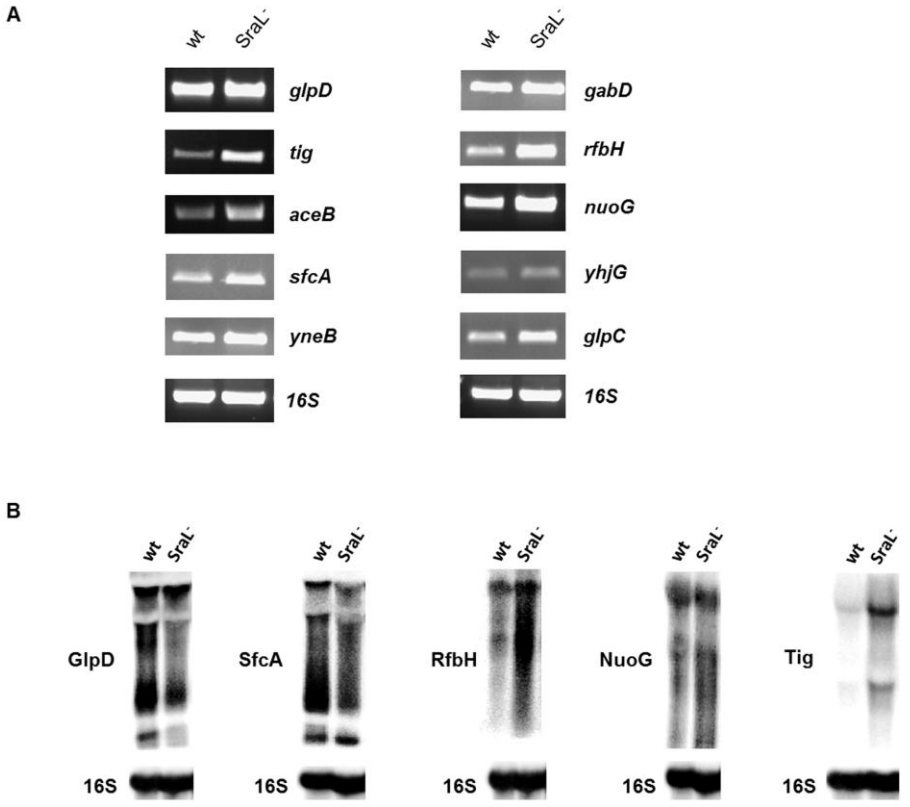

FIGURE 5 – Analysis of SraL putative targets by RT-PCR and Northern blot. Total cellular RNA was extracted from the *S.* Typhimurium strains indicated grown in LB at 37ºC till 6 h after OD_{600} of 2. **(A)** RT-PCR experiments were carried out using primers specific for the several putative targets over total RNA extracted from the wild-type and SraL deletion mutant, as indicated in each lane. RT-PCR primers specific for 16S rRNA shows that there were not significant variations in the amount of RNA used in each sample. **(B)** 15 μg of total RNA were separated on a 1.3% formaldehyde / agarose gel. The gel was then blotted to a Hybond-N^+ membrane and hybridized with the corresponding riboprobes. The membrane was stripped and then probed for 16S rRNA as loading control. GlpD: aerobic sn-glycerol-3-phosphate dehydrogenase; Tig: trigger factor; AceB: malate synthase A; SfcA: NAD-linked malate dehydrogenase; YneB: putative fructose-1,6,biphosphate aldolase; GabD: succinate-semialdehyde dehydrogenase I; RfbH: CDP-6deoxy-D-xylo-4-hexulose-3-dehydrase; NuoG: NADH dehydrogenase I chain G; YhjG: putative inner membrane protein; GlpC: anaerobic sn-glycerol-3-phosphate dehydrogenase.

Indeed, the mRNA expression level relative to some of the putative targets was also affected when comparing the wild-type with the SraL deletion mutant strain. Thus, the regulation of SraL over these putative targets seems to occur by base-pairing of the sRNA with the mRNA of its targets, that is subsequently reflected in the protein levels. This fact is in agreement with our previous results in which SraL was shown to be an Hfq-dependent sRNA (Viegas *et al.*, 2007).

The results obtained by the proteomic, RT-PCR and Northern blot analyses seem to indicate that SraL is involved directly or indirectly on the metabolism of carbohydrate in the conditions tested. Several of its putative targets perform important functions in several metabolic pathways, namely TCA or Krebs cycle (*sfcA*), oxidative phosphorylation (*nuoG*) and also the alternative glyoxylate pathway (*aceB*). Carbohydrates provide cells not only with energy but also with building blocks for synthesis of all macromolecules. It is therefore not surprising that the uptake and metabolism of carbohydrates are extensively regulated at all levels. In fact, it was already reported the existence of several sRNAs that control the sugar metabolism at various levels (for a review see (Görke and Vogel, 2008). At stationary phase cells stop growing and the metabolism slows. In this growth phase, the carbohydrates' concentration is much lower than in exponential phase. Therefore, the enzymes involved in the carbohydrates metabolism should be less needed and consequently less produced. sRNAs are key regulators that promote an efficient and fast downregulation of gene expression under specific growth conditions. SraL is possibly one of the post-transcriptional regulators of bacterial sugar pathways. Moreover, SraL sRNA seems to regulate the synthesis of the LPS O-antigen (encoded by *rfbH),* an important virulence determinant of *Salmonella* (Gantois *et al.*, 2009), which may explain the SraL expression under SPI-1 and SPI-2 inducing

conditions. This sRNA also appears to control the expression of the protein folding chaperone Trigger factor (*tig*).

Our analyses revealed the existence of several SraL putative targets, pointing the involvement of this sRNA in some common metabolic pathways in the cell. The veracity of these targets has to be confirmed with further studies. In chapter 4, the regulation of SraL over *tig* mRNA is more deeply studied.

The levels of SraL sRNA are affected by glucose

In the course of the experiments and since SraL seems to affect the expression of several enzymes related with glucose metabolism, we investigated the influence of glucose in the SraL expression levels. For this purpose, we grew *S.* Typhimurium wild-type cells until late stationary phase (OD2+6h), we then added 0.2% of glucose and several samples were collected over 30 min. In the conditions tested, the expression of SraL seemed to be dependent on glucose. Upon addition of glucose in the medium, SraL expression decreased over time (Figure 6). We used MicA sRNA as a control since this sRNA is also highly expressed in late stationary phase. In contrast with SraL, the expression of MicA sRNA remained relatively constant over time. Interestingly, Spot42 sRNA was previously shown to be indirectly regulated by glucose. This sRNA is subject to repression by the cAMP-CRP complex in the absence of glucose (Polayes *et al.*, 1988; Sahagan and Dahlberg, 1979). When glucose is present, Spot42 sRNA selectivity inhibits the synthesis of GalK, a protein encoded in the *galETKM* operon which converts galactose to the glycolytic intermediate glucose-1-phosphate (Moller *et al.*, 2002).

The biological function of SraL sRNA was not yet revealed. Along this chapter we performed the first attempts to investigate targets of this sRNA. The proteomic, RT-PCR and Northern blot analyses seem to indicate that this sRNA is

involved in the metabolism of the carbohydrates. Moreover, SraL was shown to be negatively affected by glucose. Nevertheless, it has to be further clarified whether this regulation is direct or indirect.

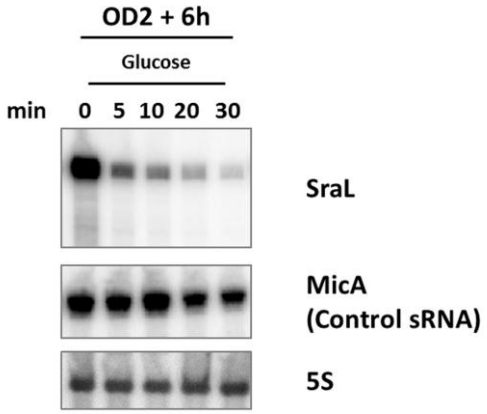

FIGURE 6 - Analysis of SraL expression in the presence of glucose. Total cellular RNA was extracted from the wild-type S. Typhimurium strain grown in LB at 37ºC till late exponential phase (OD2+6h), then glucose was added to a final concentration of 0,2% and the culture grew for more 30 min. Samples were taken at the minutes indicated in the figure. 15 μg of RNA were separated on a 6% PAA / 8.3 M urea gel. The gel was then blotted to a Hybond-N+ membrane and hybridized with the corresponding SraL and MicA riboprobe. Probing for 5S rRNA confirmed equal loading.

ACKNOWLEDGEMENTS

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REFERENCES

Abe, H., and Aiba, H. 1996. Differential contributions of two elements of rho-independent terminator to transcription termination and mRNA stabilization. *Biochimie*. 78:1035-1042.

Aiba, H., Hanamura, A., and Yamano, H. 1991. Transcriptional terminator is a positive regulatory element in the expression of the *Escherichia coli crp* gene. *J Biol Chem*. 266:1721-1727.

Amábile-Cuevas, C.F., and Demple, B. 1991. Molecular characterization of the soxRS genes of *Escherichia coli*: two genes control a superoxide stress regulon. *Nucleic Acids Res*. 19:4479-4484.

Argaman, L., Hershberg, R., Vogel, J., Bejerano, G., Wagner, E.G., Margalit, H., and Altuvia, S. 2001. Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli*. *Curr Biol*. 11:941-950.

Caron, M.P., Lafontaine, D.A., and Massé, E. 2010. Small RNA-mediated regulation at the level of transcript stability. *RNA Biol*. 7:140-144.

Datsenko, K.A., and Wanner, B.L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A*. 97:6640-6645.

Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., and Van Immerseel, F. 2009. The *Salmonella* Enteritidis lipopolysaccharide biosynthesis gene *rfbH* is required for survival in egg albumen. *Zoonoses Public Health*. 56:145-149.

García-del Portillo, F., Calvo, E., D'Orazio, V., and Pucciarelli, M.G. 2011. Association of ActA to peptidoglycan revealed by cell wall proteomics of intracellular *Listeria monocytogenes*. *J Biol Chem*. 286:34675-34689.

Görke, B., and Vogel, J. 2008. Noncoding RNA control of the making and breaking of sugars. *Genes Dev*. 22:2914-2925.

Hoiseth, S.K., and Stocker, B.A. 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature*. 291:238-239.

Lober, S., Jackel, D., Kaiser, N., and Hensel, M. 2006. Regulation of *Salmonella* pathogenicity island 2 genes by independent environmental signals. *Int J Med Microbiol*. 296:435-447.

Lutz, R., and Bujard, H. 1997. Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. *Nucleic Acids Res*. 25:1203-1210.

Massé, E., Majdalani, N., and Gottesman, S. 2003. Regulatory roles for small RNAs in bacteria. *Curr Opin Microbiol*. 6:120-124.

Moller, T., Franch, T., Udesen, C., Gerdes, K., and Valentin-Hansen, P. 2002. Spot 42 RNA mediates discoordinate expression of the *E. coli* galactose operon. *Genes Dev*. 16:1696-1706.

Ortega, A.D., Gonzalo-Asensio, J., and García-Del Portillo, F. 2012. Dynamics of *Salmonella* small RNA expression in non-growing bacteria located inside eukaryotic cells. *RNA Biol*. 9.

Polayes, D.A., Rice, P.W., Garner, M.M., and Dahlberg, J.E. 1988. Cyclic AMP-cyclic AMP receptor protein as a repressor of transcription of the spf gene of *Escherichia coli*. *J Bacteriol*. 170:3110-3114.

Rasmussen, A.A., Eriksen, M., Gilany, K., Udesen, C., Franch, T., Petersen, C., and Valentin-Hansen, P. 2005. Regulation of *ompA* mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. *Mol Microbiol*. 58:1421-1429.

Sahagan, B.G., and Dahlberg, J.E. 1979. A small, unstable RNA molecule of *Escherichia coli*: Spot 42 RNA. II. Accumulation and distribution. *J Mol Biol*. 131:593-605.

Schmieger, H. 1971. The fate of the bacterial chromosome in P22-infected cells of *Salmonella typhimurium*. *Mol Gen Genet*. 110:238-244.

Sittka, A., Lucchini, S., Papenfort, K., Sharma, C.M., Rolle, K., Binnewies, T.T., Hinton, J.C., and Vogel, J. 2008. Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq. *PLoS Genet*. 4:e1000163.

Sittka, A., Pfeiffer, V., Tedin, K., and Vogel, J. 2007. The RNA chaperone Hfq is essential for the virulence of *Salmonella typhimurium*. *Mol Microbiol*. 63:193-217.

Sridhar, J., Sambaturu, N., Sabarinathan, R., Ou, H.Y., Deng, Z., Sekar, K., Rafi, Z.A., and Rajakumar, K. 2010. sRNAscanner: a computational tool for intergenic small RNA detection in bacterial genomes. *PLoS One*. 5:e11970.

Storz, G., Vogel, J., and Wassarman, K.M. 2011. Regulation by small RNAs in bacteria: expanding frontiers. *Mol Cell*. 43:880-891.

Udekwu, K.I., Darfeuille, F., Vogel, J., Reimegard, J., Holmqvist, E., and Wagner, E.G. 2005. Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. *Genes Dev*. 19:2355-2366.

Viegas, S.C., Pfeiffer, V., Sittka, A., Silva, I.J., Vogel, J., and Arraiano, C.M. 2007. Characterization of the role of ribonucleases in *Salmonella* small RNA decay. *Nucleic Acids Res*. 35:7651-7664.

Vogel, J., Bartels, V., Tang, T.H., Churakov, G., Slagter-Jager, J.G., Huttenhofer, A., and Wagner, E.G. 2003. RNomics in *Escherichia coli* detects new sRNA species and indicates parallel transcriptional output in bacteria. *Nucleic Acids Res*. 31:6435-6443.

Wassarman, K.M., Repoila, F., Rosenow, C., Storz, G., and Gottesman, S. 2001. Identification of novel small RNAs using comparative genomics and microarrays. *Genes Dev*. 15:1637-1651.

Waters, L.S., and Storz, G. 2009. Regulatory RNAs in bacteria. *Cell*. 136:615-628.

Zhang, A., Wassarman, K.M., Rosenow, C., Tjaden, B.C., Storz, G., and Gottesman, S. 2003. Global analysis of small RNA and mRNA targets of Hfq. *Mol Microbiol*. 50:1111-1124.

Zuker, M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*. 31:3406-3415.

SUPPLEMENTARY INFORMATION

Supplementary Tables

SUPPLEMENTARY TABLE S1 - List of oligonucleotides used in this work. The restriction sequences in the primers used for cloning procedures are shown in bold. T7 sequences in the primers used for riboprobe synthesis are underlined.

Oligo	Sequence 5' to 3'
pIS-001	ATTTCGGCTAAAAAATCAGCATTTCGCTGGCGAACAGGGCGTCGTCGCTTAGTGTAGGCTGGAGCTGCTTC
pIS-002	CATGCACTCGGCCATCGGGCTGAGCTCACCTAAAACTAAAGCGCCGCTAAGTGGTCCATATGAATATCCTCCTTAG
pIS-003	GTTTTTCTCGAGATGCGCTGCGAAC
pIS-004	GTTTTTTCTAGAAGATGATTAACATGCACTCG
pIS-009	ATCAACACAAACCGGAACCTC
pIS-010	**GTTTTTGGTACC**CACTCGGCCATCGGGCTG
pIS-016 (*tig*-RT1)	CAGCTCCAGAGCCTGTTTC
pIS-017 (*tig*-RT2)	GAAGAGTTCGAAGGCGGCAAAG
pIS-018	AGGCGGTCTGTCAAGTCGGATG
pIS-019	ACAGCCATGCAGCACCTGTCTC
pIS-021	ATCAACACAAACCGGAAC
pIS-022	GTTTTTTTTTAATACGACTCACTATAGGGAGGTAAGGGCGCTTTAGTTTG
pIS-023	CTACGGCGTTTCACTTCTGAGTTC
pIS-025 (*rfbH*-RT1)	AGCGAGCTCTCAAACCTGGTG
pIS-026 (*rfbH*-RT2)	GAGGAAGTGATCCCAATTGCTG
pIS-027 (*nuoG*-RT1)	GGTGAGGATGTCACTCAGACC
pIS-028 (*nuoG*-RT2)	CGGGCAATCATAGTAATACCG
pIS-029 (*aceB*-RT1)	AAAGCGCTGCTCGCTAAAGG
pIS-030 (*aceB*-RT2)	ATGCGCAATCCACGTGCCGTC
pIS-031 (*glpC*-RT1)	TGCATCCAGTTTGCGCCATAG
pIS-032 (*glpC*-RT2)	AAGCCAGTGCGCCAGTTGCTTG
pIS-033 (*sfcA*-RT1)	CTTCCCGTTGTGCTGGATGTC
pIS-034 (*sfcA*-RT2)	GCTGGAGATTGTCGCATTTCTG
pIS-035 (*glpD*-RT1)	TATGTACGATCACCTCGGCAAACG
pIS-036 (*glpD*-RT2)	CCTGCTTCTGATTATGGACG
pIS-037 (*yhjG*-RT1)	TATGCTGGCGCTACGCAGCGAAAG
pIS-038 (*yhjG*-RT2)	GTTTCGACTGCTCAGCGTCTTTC
pIS-039 (*yneB*-RT1)	GCGCCGCTCTTTGAATATGCTG
pIS-040 (*yneB*-RT2)	CCAATGCTTCACGTTCAGGC
pIS-041(*gabD*-RT1)	CTGCTGGTGATTAAACAGCCC
pIS-042 (*gabD*-RT2)	CGCCGTCCTGAACATACAATC
pIS-043	GTTTTTTTTTAATACGACTCACTATAGGGAGGCACGGAGTGGCCAAA
pIS-044	GAAAGACGCGCATTTGT

Oligo	Sequence 5' to 3'
pIS-045	TATGTACGATCACCTCGGCAAACG
pIS-046	GTTTTTTTTTTAATACGACTCACTATAGGGTCGATATC TTCCGCTTCGAC
pIS-047	CTTCCCGTTGTGCTGGATGTC
pIS-048	GTTTTTTTTTTAATACGACTCACTATAGGGGAGCAAAT CTCATCACGATAGC
pIS-049	AGCGAGCTCTCAAACCTGGTG
pIS-050	GTTTTTTTTTTAATACGACTCACTATAGGGGCATCACAGCAGTCTTCAATC
pIS-051	GGTGAGGATGTCACTCAGACC
pIS-052	GTTTTTTTTTTAATACGACTCACTATAGGATATCATCCAGACGGGTGTCATCC
pIS-054	GTTTTTTTTTTAATACGACTCACTATAGGGTCAGTTCC GGCAGTTCGCGTTCTTC

SUPPLEMENTARY TABLE S2 - List of the proteins analysed by proteomic analysis in wild-type (wt), SraL deletion mutant (dSraL) and SraL overexpressing strain (pSraL).

#	Identified Proteins (713)	Accession	pSraL	WT	dSraL
1	new outer membrane protein predicted bacterial porin (nmpC)	STM1572	13	13	13
2	protein chain elongation factor EF-Tu (duplicate of (tufA)	STM3445	22	19	21
3	putative hydrogenase, membrane component (ompA) .	STM1070	16	17	18
4	outer membrane protein 1b (ibc), porin (ompC)	STM2267	13	15	17
5	chaperone Hsp60 with peptide-dependent ATPase activity (mopA)	STM4330	27	30	31
6	flagellar biosynthesis flagellin, filament structural protein (fliC)	STM1959	26	24	24
7	glycerol kinase (glpK)	STM4086	25	29	28
8	stress response DNA-binding protein starvation (dps)	STM0831	15	13	15
9	outer membrane protein W colicin S4 receptor; (ompW)	STM1732	4	4	6
10	putative cytoplasmic protein (yciF)	STM1729	9	10	9
11	malate dehydrogenase (mdh)	STM3359	15	18	16
12	phosphoglyceromutase 1 (gpmA)	STM0772	9	10	13
13	outer membrane protease, receptor for phage OX2 (ompX)	STM0833	11	11	10
14	glycerophosphodiester phosphodiesterase, periplasmic (glpQ)	STM2282	17	20	18
15	cell invasion protein (sipC)	STM2884	18	21	21
16	chaperone Hsp70 in DNA biosynthesis/cell division (dnaK)	STM0012	35	30	29
17	phosphoglycerate kinase (pgk)	STM3069	19	17	19
18	50S ribosomal subunit protein L5 (rplE)	STM3428	10	10	9
19	enolase (eno)	STM2952	15	12	15
20	sn-glycerol-3-phosphate dehydrogenase (aerobic) (glpD)	STM3526	18	21	25
21	peptidyl-prolyl cis/trans isomerase, trigger factor a molecular (tig)	STM0447	11	14	20
22	2-oxoglutarate dehydrogenase (sucB)	STM0737	6	7	7
23	phosphoenolpyruvate carboxykinase (pckA)	STM3500	20	19	18
24	catalase hydroperoxidase HPII(III), RpoS dependent (katE)	STM1318	20	17	18
25	membrane-bound ATP synthase, F1 sector, beta-subunit (atpD)	STM3865	15	15	19
26	ATP-dependent protease, Hsp 100, part of novel (clpB)	STM2660	28	26	28
27	GTP cyclohydrolase I (folE)	STM2193	2	0	0
28	glyceraldehyde-3-phosphate dehydrogenase A (gapA)	STM1290	14	16	14
29	30S ribosomal subunit protein S7, initiates assembly (rpsG)	STM3447	11	9	11
30	PhoP regulated: reduced macrophage survival (pagC)	STM1246	9	10	10
31	putative NAD-dependent aldehyde dehydrogenase	STM4519	20	15	17
32	pyruvate dehydrogenase, decarboxylase component (aceE)	STM0152	20	22	24
33	aconitate hydratase 2 (acnB)	STM0158	21	20	27
34	protein chain elongation factor EF-G, GTP-binding (fusA)	STM3446	19	20	19
35	30S ribosomal subunit protein S9 (rpsI)	STM3344	5	3	5
36	citrate synthase (gltA) [4.1.3.7]	STM0730	18	14	14
37	50S ribosomal subunit protein L22 (rplV)	STM3435	10	8	9
38	30S ribosomal subunit protein S4 (rpsD)	STM3416	12	15	14
39	aldehyde dehydrogenase B (lactaldehyde dehydrogenase) (aldB)	STM3680	10	13	11
40	alcohol dehydrogenase, propanol preferring (adhP)	STM1567	10	10	10
41	50S ribosomal subunit protein L1, regulates synthesis (rplA)	STM4150	10	11	9
42	iron-dependent alcohol dehydrogenase (adhE)	STM1749	18	23	20
43	chaperone Hsp90, heat shock protein C 62.5 (htpG)	STM0487	20	21	21
44	aspartate ammonia-lyase (aspartase) (aspA)	STM4326	12	16	17
45	membrane-bound ATP synthase, F1 sector, alpha-subunit (atpA)	STM3867	13	12	16
46	3-oxoacyl-[acyl-carrier-protein] synthase I (fbaB)	STM2141	15	13	12
47	putative catalase .	STM1731	14	11	12
48	fructose-bisphosphate aldolase (fba)	STM3068	8	8	10
49	malate synthase A (aceB)	STM4183	7	10	17
50	aconitate hydratase 1 (acnA)	STM1712	17	15	18

51	superoxide dismutase, iron (sodB)	STM1431	4	4	4
52	putative Ser protein kinase (yeaG)	STM1285	15	17	16
53	putative oxidoreductase (yghA)	STM3157	10	11	10
54	30S ribosomal subunit protein S2 (rpsB)	STM0216	12	12	11
55	cell invasion protein (sipA)	STM2882	16	21	14
56	Salmonella outer protein: homologous to ipgD of (sopB)	STM1091	13	19	14
57	sensory histitine protein kinase, transduces signal between (cheA)	STM1921	11	13	11
58	putative cytoplasmic protein (yciE)	STM1730	9	9	7
59	ABC superfamily (periplasm)(oppA)	STM1746	14	14	13
60	flagellar biosynthesis filament capping protein (fliD)	STM1960	15	15	13
61	2-oxoglutarate dehydrogenase (decarboxylase component) (sucA)	STM0736	14	19	15
62	50S ribosomal subunit protein L19 (rplS)	STM2673	5	5	5
63	succinyl-CoA synthetase, beta subunit (sucC)	STM0738	13	13	14
64	bacterioferrin, an iron storage homoprotein (bfr)	STM3443	5	5	4
65	hyperosmotically inducible periplasmic protein (osmY)	STM4561	9	6	7
66	RNA polymerase, alpha subunit (rpoA)	STM3415	10	9	9
67	isocitrate dehydrogenase in e14 prophage, specific for (icdA)	STM1238	9	10	12
68	response regulator in two-component regulatory system (phoP)	STM1231	7	8	8
69	isocitrate lyase (aceA)	STM4184	13	7	15
70	glutathionine S-transferase (gst)	STM1451	10	8	9
71	cell invasion protein (sipB)	STM2885	11	15	13
72	putative cytoplasmic protein (yaeH)	STM0211	9	7	9
73	DNA-binding, ATP-dependent protease la cleaves RcsA and (lon)	STM0450	10	14	12
74	transaldolase A (talA)	STM2473	7	12	12
75	glutaredoxin 2 (grxB)	STM1165	9	11	10
76	transaldolase B (talB)	STM0007	8	9	9
77	30S ribosomal subunit protein S5 (rpsE)	STM3423	4	5	5
78	NAD-linked malate dehydrogenase (sfcA)	STM1566	9	10	15
79	succinate dehydrogenase, flavoprotein subunit (sdhA)	STM0734	12	14	11
80	putative thiol - alkyl hydroperoxide reductase	STM0402	6	7	5
81	methyl accepting chemotaxis protein II (cheM)	STM1919	13	15	11
82	putative molecular chaperone (small heat shock protein)	STM1251	7	6	6
83	trehalase, periplasmic (treA)	STM1796	13	12	12
84	RNA polymerase, beta subunit (rpoB)	STM4153	8	18	14
85	conjugative transfer: surface exclusion (traT)	PSLT103	5	6	6
86	gluconate-6-phosphate dehydrogenase, decarboxylating (gnd)	STM2081	12	11	16
87	pyruvate dehydrogenase (aceF)	STM0153	10	14	12
88	putative NADP-dependent oxidoreductase (yncB)	STM1589	8	8	6
89	succinate dehydrogenase, Fe-S protein (sdhB)	STM0735	6	8	5
90	phosphoenolpyruvate synthase (pps)	STM1349	12	12	13
91	50S ribosomal subunit protein L9 (rplI)	STM4394	6	6	5
92	50S ribosomal subunit protein L13 (rplM)	STM3345	9	7	7
93	chaperone Hsp10, affects cell division (mopB)	STM4329	4	4	4
94	putative fructose-1,6-bisphosphate aldolase (yneB)	STM4078	9	8	5
95	putative ABC superfamily (atp_bind) transport protein (yjjK)	STM4581	13	11	12
96	flagellar biosynthesis, hook-filament junction protein 1 (flgK)	STM1183	11	11	12
97	DNA-binding protein HLP-II (HU, BH2, HD, NS) (hns)	STM1751	5	7	7
98	adenylosuccinate synthetase (purA)	STM4366	11	12	11
99	pyruvate formate lyase I, induced anaerobically (pflB)	STM0973	9	13	13
100	succinyl-CoA synthetase, alpha subunit (sucD)	STM0739	8	7	8
101	putative ABC superfamily (peri_perm) (yneA)	STM4077	11	11	9
102	50S ribosomal subunit protein L4, regulates expression (rplD)	STM3439	4	6	4
103	lipoamide dehydrogenase (NADH) (lpdA)	STM0154	6	10	9

104	glycine tRNA synthetase, beta subunit (glyS)	STM3655	10	11	8
105	DNA-binding protein with chaperone activity (stpA)	STM2799	8	7	9
106	lysine tRNA synthetase, constitutive (lysS)	STM3040	8	13	9
107	3-deoxy-D-manno-octulosonic acid 8-P synthetase (kdsA)	STM1772	8	9	8
108	uracil phosphoribosyltransferase (upp)	STM2498	7	7	7
109	transcription termination factor Rho polarity suppressor (rho)	STM3917	7	9	7
110	fumarase C (fumarate hydratase Class II) (fumC)	STM1469	8	10	6
111	Salmonella iron transporter: fur regulated (sitA)	STM2861	5	5	3
112	outer membrane channel specific tolerance to colicin (tolC)	STM3186	12	8	9
113	sn-glycerol 3-phosphate transport protein (ugpB)	STM3557	9	10	12
114	putative LysM domain (ygaU)	STM2795	9	6	7
115	aspartate aminotransferase (aspC)	STM0998	8	11	10
116	NADH dehydrogenase I chain C,D (nuoC)	STM2326	10	8	8
117	putative periplasmic protein (ydgA)	STM1466	9	8	8
118	50S ribosomal subunit protein L16 (rplP)	STM3433	4	4	4
119	membrane-bound ATP synthase, F0 sector, subunit b (atpF)	STM3869	11	9	8
120	30S ribosomal subunit protein S13 (rpsM)	STM3418	6	6	6
121	trp-repressor binding protein (wraB)	STM1119	6	6	6
122	sn-glycerol-3-phosphate dehydrogenase (anaerobic) (glpA)	STM2284	4	9	8
123	50S ribosomal subunit protein L6 (rplF)	STM3425	10	9	10
124	superoxide dismutase, manganese (sodA)	STM4055	8	5	7
125	putative methyl-accepting chemotaxis protein	STM3138	3	8	8
126	phosphoglucomutase (pgm)	STM0698	6	7	7
127	fumarate reductase, anaerobic, flavoprotein subunit (frdA)	STM4343	9	8	9
128	putative resistance protein, osmotically inducible (osmC)	STM1563	7	7	6
129	putative inner membrane protein (elaB)	STM2311	0	3	3
130	aminoacyl-histidine dipeptidase (peptidase D) (pepD)	STM0316	9	7	7
131	valine tRNA synthetase (valS)	STM4475	6	4	6
132	phosphopentomutase (deoB)	STM4569	9	5	5
133	pyruvate kinase I (formerly F), fructose stimulated (pykF)	STM1378	8	9	10
134	putative cytoplasmic protein (yajQ)	STM0435	7	7	7
135	succinate-semialdehyde dehydrogenase I (gabD)	STM2791	11	7	5
136	ATP-dependent zinc-metallo protease (hflB)	STM3296	8	8	8
137	acetyl-CoA synthetase (acs)	STM4275	8	11	10
138	50S ribosomal subunit protein L3 (rplC)	STM3440	6	5	5
139	isoaspartyl dipeptidase (iadA)	STM4512	6	6	5
140	putative ABC superfamily transport protein (possibly (yehZ)	STM2165	6	7	6
141	glutamate tRNA synthetase, catalytic subunit (gltX)	STM2415	7	8	7
142	nucleoside channel receptor of phage T6 and (tsx)	STM0413	6	4	8
143	3-oxoacyl-[acyl-carrier-protein] synthase I (fabB)	STM2378	7	8	8
144	30S ribosomal subunit protein S1 (rpsA)	STM0981	5	9	3
145	glucosephosphate isomerase (pgi)	STM4221	7	6	6
146	PTS family, glucose-specific IIA component (crr)	STM2433	5	7	8
147	50S ribosomal subunit protein L10 (rplJ)	STM4151	4	4	4
148	50S ribosomal subunit protein L24 (rplX)	STM3429	6	7	7
149	putative outer membrane antigen (yaeT)	STM0224	9	7	13
150	CDP-6deoxy-D-xylo-4-hexulose-3-dehydrase (rfbH)	STM2090	5	8	10
151	ABC superfamily (peri_perm), galactose transport protein (mglB)	STM2190	6	4	7
152	tol protein required for outer membrane integrity (pal)	STM0749	5	5	5
153	putative inner membrane protein (ygaM)	STM2802	3	2	3
154	transcription pausing L factor (nusA)	STM3287	7	8	5
155	CTP synthetase (pyrG)	STM2953	5	8	6

156	alkyl hydroperoxide reductase (ahpC)	STM0608	5	5	5
157	D-amino acid dehydrogenase subunit (dadA)	STM1803	3	4	4
158	ABC superfamily (peri_perm), dipeptide transport protein (dppA)	STM3630	7	5	8
159	peptidyl-prolyl cis-trans isomerase, survival protein (surA)	STM0092	7	9	7
160	with HflK, part of modulator for protease (hflC)	STM4364	5	5	6
161	subunit of cysteine synthase A and O-acetylserine (cysK)	STM2430	10	7	6
162	protein chain elongation factor EF-Ts (tsf)	STM0217	9	5	8
163	proline tRNA synthetase (proS)	STM0242	5	6	7
164	General PTS family PEP-protein phosphotransferase (ptsI)	STM2432	4	7	8
165	thiol peroxidase (tpx)	STM1682	5	6	6
166	transketolase 2, isozyme (tktB)	STM2474	6	7	7
167	ABC superfamily glutamine high-affinity transporter (glnH)	STM0830	8	7	6
168	flagellar biosynthesis hook-filament junction protein (flgL)	STM1184	7	6	5
169	transport of long-chain fatty acids sensitivity to (fadL)	STM2391	8	8	8
170	oligopeptidase A (prlC)	STM3594	6	11	8
171	NADH dehydrogenase I chain G (nuoG)	STM2323	4	7	10
172	phenylalanine tRNA synthetase, beta-subunit (pheT)	STM1338	4	5	7
173	adenylate kinase (adk)	STM0488	4	5	6
174	serine tRNA synthetase also charges selenocystein (serS)	STM0963	7	8	8
175	phosphotransacetylase (pta)	STM2338	5	7	9
176	L-lactate dehydrogenase (lldD)	STM3694	7	8	7
177	phage lambda receptor maltose high-affinity receptor (lamB)	STM4231	6	8	7
178	50S ribosomal subunit protein L15 (rplO)	STM3421	4	5	5
179	50 S ribosomal subunit protein L11 (rplK)	STM4149	3	4	5
180	galactose-1-epimerase (mutarotase) (galM)	STM0773	3	0	3
181	50S ribosomal subunit protein L2 (rplB)	STM3437	3	4	6
182	NAD synthetase, prefers NH3 over glutamine (nadE)	STM1310	6	7	8
183	putative glutathione S-transferase (yibF)	STM3684	6	5	6
184	with HflC, part of modulator for protease (hflK)	STM4363	7	7	8
185	riboflavin synthase, beta chain (ribH)	STM0417	5	6	5
186	response regulator in two-component regulatory (arcA)	STM4598	6	5	7
187	putative Universal stress protein UspA and related (ybdQ)	STM0614	4	5	4
188	universal stress protein A (uspA)	STM3591	5	3	5
189	putative chemotaxis signal transduction protein	STM2314	2	7	6
190	methyl-accepting chemotaxis protein I, serine sensor receptor (tsr)	STM4533	2	7	7
191	putative outer membrane lipoprotein (yaeC)	STM0245	8	6	7
192	leucine tRNA synthetase (leuS)	STM0648	6	8	7
193	ABC superfamily, spermidine/putrescine transporter (potD)	STM1222	7	8	6
194	2,5-diketo-D-gluconate reductase A (yqhE)	STM3165	6	8	6
195	serine endoprotease (degQ)	STM3348	7	6	5
196	putative aldehyde dehydrogenase (ydcW)	STM1597	7	5	7
197	membrane-bound ATP synthase, gamma-subunit (atpG)	STM3866	5	7	5
198	ABC superfamily maltose transport protein, substrate (malE) .	STM4229	4	5	6
199	asparagine tRNA synthetase (asnS)	STM1000	4	10	8
200	murein lipoprotein, links outer and inner membranes (lpp)	STM1377	3	3	3
201	molecular chaparone heat shock protein (grpE)	STM2681	3	2	2
202	aminopeptidase A (pepA)	STM4477	4	5	4
203	response regulator in two-component regulatory system (ompR)	STM3502	6	5	7
204	isoleucine tRNA synthetase (ileS)	STM0046	4	7	8
205	3-oxoacyl-[acyl-carrier-protein] reductase (fabG)	STM1195	6	6	7
206	putative inner membrane lipoprotein	STM3580	5	6	6
207	Organic solvent tolerance protein (imp)	STM0093	8	5	8

208	ABC superfamily (bind_prot), histidine transport protein (hisJ)	STM2354	6	6	5
209	acetylCoA carboxylase, carboxytransferase component (accA)	STM0232	5	5	6
210	scaffolding protein for murein-synthesizing holoenzyme (mipA)	STM1286	3	5	4
211	stringent starvation protein A, regulator of transcription (sspA)	STM3342	4	4	5
212	ribosome releasing factor (frr)	STM0219	5	6	6
213	ribonucleoside diphosphate reductase 1, alpha subunit (nrdA)	STM2277	3	9	6
214	dihydrodipicolinate synthase (dapA)	STM2489	7	5	7
215	ADP-L-glycero-D-mannoheptose-6-epimerase (rfaD)	STM3710	6	7	6
216	SLP3_0001 undefined product 30:842 reverse MW:28469	SLP3_0001	5	8	7
217	invasion protein outer membrane (invG)	STM2898	6	6	5
218	NADH dehydrogenase I chain F (nuoF)	STM2324	5	5	7
219	30S ribosomal subunit protein S3 (rpsC)	STM3434	5	6	6
220	transketolase 1 isozyme (tktA)	STM3076	4	5	6
221	glycoprotein/polysaccharide metabolism (ybaY)	STM0465	2	3	2
222	ATPase component of the HslUV protease (hslU)	STM4091	6	5	6
223	tubulin-like GTP-binding protein and GTPase, (ftsZ)	STM0133	4	8	7
224	tol protein required for outer membrane integrity (tolB)	STM0748	5	3	6
225	fumarate reductase, anaerobic, Fe-S protein subunit (frdB)	STM4342	5	3	6
226	triosephosphate isomerase (tpiA)	STM4081	3	4	3
227	putative cytoplasmic protein (yfbU)	STM2335	4	3	4
228	putative periplasmic binding transport protein (fliY)	STM1954	5	5	3
229	DNA gyrase, subunit B (type II topoisomerase) (gyrB)	STM3835	2	6	4
230	putative methyl-accepting chemotaxis protein	STM3216	4	7	6
231	lipoprotein-34 (nlpB)	STM2488	6	6	6
232	putative cytoplasmic protein (yjgA)	STM4437	7	7	6
233	component in transcription antitermination (nusG)	STM4148	6	6	5
234	putative oxidoreductase (ydjA)	STM1296	6	5	4
235	putative universal stress protein (ynaF)	STM1652	6	4	4
236	Sugar Specific PTS family, mannose-specific enzyme IIAB (manX)	STM1830	3	5	4
237	glucose-6-phosphate dehydrogenase (zwf)	STM1886	5	6	7
238	trehalose-6-phosphate synthase (otsA)	STM1928	7	5	8
239	response regulator in two-component regulatory system (rcsB)	STM2270	5	8	6
240	catabolite activator protein (CAP), cyclic AMP receptor (crp)	STM3466	4	4	7
241	periplasmic L-asparaginase II (ansB)	STM3106	5	3	3
242	putative sulfurtransferase (sseA)	STM2533	4	4	6
243	N-succinyltransferase (dapD)	STM0213	4	6	3
244	enoyl-[acyl-carrier-protein] reductase (NADH) (fabI)	STM1700	3	5	4
245	putative serine/threonine protein kinase (yfgL)	STM2520	4	4	6
246	protein chain initiation factor IF-2 (infB)	STM3286	0	8	5
247	3-hydroxyacyl-coA dehydrogenase of 4-enzyme FadB (fadB)	STM3983	3	5	4
248	phosphoribosylpyrophosphate synthetase (prsA)	STM1780	6	6	5
249	peptidyl prolyl isomerase (cypD)	STM0452	5	6	6
250	cell division inhibitor, a membrane ATPase, activates (minD)	STM1815	4	4	5
251	6-phosphofructokinase II (pfkB)	STM1326	5	8	5
252	acetyl CoA carboxylase, biotin carboxylase subunit (accC)	STM3380	4	4	6
253	putative periplasmic protein (yhcB)	STM3347	4	6	6
254	4-aminobutyrate aminotransferase (gabT)	STM2792	5	4	5
255	SLP2_0019 undefined product 14223:14852 forward MW:22398	SLP2_0019	4	5	3
256	30S ribosomal subunit protein S6 (rpsF)	STM4391	4	4	3
257	aminopeptidase N (pepN)	STM1057	3	5	6
258	alanyl-tRNA synthetase (alaS)	STM2827	3	8	7
259	50S ribosomal subunit protein L7/L12 (rplL)	STM4152	3	4	3
260	putative formate dehydrogenase formation protein ? Mn_fn (fdhE)	STM4034	2	2	2

261	putative oxidoreductase (ydfG)	STM1511	5	3	4
262	putative Histidinol phosphatase and related hydrolases of (ycdX)	STM1136	3	2	4
263	acyl carrier protein (acpP) .	STM1196	2	2	2
264	(3R)-hydroxymyristol acyl carrier protein dehydratase (fabZ)	STM0227	5	2	3
265	putative glutathionylspermidine synthase (ygiC)	STM3188	6	4	4
266	FKBP-type peptidyl-prolyl cis-trans isomerase (rotamase) (fkpA)	STM3453	4	2	3
267	tricarboxylic transport	STM2786	5	6	5
268	glucose-1-phosphatase (agp)	STM1117	5	5	4
269	putative transcriptional repressor (IclR family) (kdgR)	STM1842	5	5	5
270	mannose-6-phosphate isomerase (manA)	STM1467	5	4	5
271	paral putative transferase (maeB)	STM2472	3	6	4
272	thioredoxin reductase (trxB)	STM0958	4	5	3
273	putative inner membrane protein (yhjG)	STM3610	1	4	8
274	putative outer membrane lipoprotein (slyB)	STM1445	5	4	1
275	putative aminopeptidase (pepB)	STM2536	4	6	5
276	putative ABC superfamily transport protein (yrbC)	STM3310	5	6	5
277	acridine efflux pump (acrA)	STM0476	5	4	6
278	putative ManNAc-6P epimerase (nanE)	STM3337	4	4	5
279	sn-glycerol-3-phosphate dehydrogenase (anaerobic) (glpB)	STM2285	4	4	4
280	putative universal stress protein (ydaA)	STM1661	5	3	3
281	2-deoxyribose-5-phosphate aldolase (deoC)	STM4567	2	4	5
282	heat shock protein, DnaJ and GrpE stimulates (dnaJ)	STM0013	5	3	5
283	peptidyl-prolyl cis-trans isomerase B (rotamase B) (ppiB)	STM0536	3	4	3
284	Flagellar synthesis: phase 2 flagellin (filament structural (fljB)	STM2771	3	3	0
285	sigma D (sigma 70) factor of RNA (rpoD)	STM3211	3	5	5
286	methionine tRNA synthetase (metG)	STM2155	4	5	5
287	arginine tRNA synthetase (argS)	STM1909	0	5	4
288	outer membrane protein 1a porin (ompF)	STM0999	4	5	3
289	affects pool of 3-phosphoadenosine-5- (cysQ)	STM4404	4	4	5
290	ABC superfamily, glutamate/aspartate transporter (gltI)	STM0665	4	3	3
291	agmatinase (speB)	STM3078	4	4	4
292	SLP3_0014 undefined product 7854:8654 reverse MW:29568	SLP3_0014	5	3	4
293	succinylornithine transaminase (astC)	STM1303	3	6	6
294	putative GTP-binding protein (ychF)	STM1784	4	4	3
295	methyl-accepting chemotaxis protein III, ribose and galactose (trg)	STM1626	3	6	5
296	putative cytoplasmic protein	STM1624	3	5	5
297	putative inner membrane protein (yebE)	STM1880	6	4	3
298	regulator for lrp regulon and high-affinity branched-chain (lrp)	STM0959	4	5	3
299	putative intracellular proteinase (yhbO)	STM3269	3	3	3
300	putative thiamine pyrophosphate enzymes	STM2405	5	4	2
301	beta-D-glucoside glucohydrolase, periplasmic (bglX)	STM2166	8	2	5
302	paral putative periplasmic protein (yraP)	STM3267	3	5	3
303	50S ribosomal subunit protein L14 (rplN)	STM3430	4	2	2
304	paral putative transglycosylase (yraM)	STM3264	4	2	7
305	periplasmic glucans biosynthesis protein (mdoG)	STM1150	6	5	4
306	tyrosine tRNA synthetase (tyrS)	STM1449	5	4	4
307	putative oxidoreductase (ucpA)	STM2445	4	5	5
308	fructose-bisphosphatase (fbp)	STM4415	4	4	4
309	transcriptional regulator of cryptic csgA gene for (crl)	STM0319	4	4	4
310	putative glutathione S-transferase (yqjG)	STM3233	5	3	4
311	50S ribosomal subunit protein L17 (rplQ)	STM3414	3	4	4
312	putative cytoplasmic protein (ycbG)	STM1069	6	3	6
313	bifunctional putative sugar nucleotide transferase domain of (rfaE)	STM3200	3	4	4

314	putative inner membrane protein (ydcF)	STM1640	2	3	3
315	molybdopterin biosynthesis, protein B (moaB)	STM0803	4	3	3
316	protein chain initiation factor IF-3 (infC)	STM1334	4	3	3
317	threonine tRNA synthetase (thrS)	STM1333	2	5	6
318	glycine cleavage complex protein P, glycine decarboxylase (gcvP)	STM3053	2	5	5
319	periplasmic serine protease Do, heat shock protein (htrA)	STM0209	5	3	2
320	polynucleotide phosphorylase (pnp)	STM3282	1	6	4
321	phage shock protein negative regulatory gene for (pspA)	STM1690	0	2	3
322	purine-nucleoside phosphorylase (deoD)	STM4570	3	3	2
323	fumarase A (fumarate hydratase class I), aerobic (fumA)	STM1468	3	5	5
324	CDP-diacylglycerol phosphotidylhydrolase (ushB)	STM4064	4	4	5
325	acetate kinase A (propionate kinase 2) (ackA)	STM2337	4	4	4
326	putative oxidoreductase	STM2406	4	4	4
327	putative outer membrane lipoprotein	STM1607	3	4	5
328	2-amino-3-ketobutyrate CoA ligase (glycine acetyltransferase) (kbl)	STM3709	4	5	3
329	inorganic pyrophosphatase (ppa)	STM4414	4	3	3
330	putative aminomethyltransferase (ygfZ)	STM3048	4	6	3
331	putative glutathione S-transferase (yliJ)	STM0862	3	4	4
332	putative ABC exporter outer membrane component homolog	STM4259	3	5	3
333	putative cytoplasmic protein	STM1672	3	5	3
334	putative carboxyphosphonoenolpyruvate mutase (prpB)	STM0368	3	5	3
335	quinone oxidoreductase, NADPH dependent (qor)	STM4245	3	3	3
336	ABC superfamily high-affinity phosphate transporter (pstS)	STM3857	5	2	5
337	putative cytoplasmic protein	STM2789	5	5	2
338	putative esterase (ycfP)	STM1210	2	3	4
339	ribonucleoside-diphosphate reductase 1, beta subunit (nrdB)	STM2278	3	2	4
340	putative periplasmic immunogenic protein (yggE)	STM3065	5	0	4
341	sn-glycerol-3-phosphate dehydrogenase (anaerobic) (glpC)	STM2286	0	2	9
342	amidophosphoribosyltransferase (PRPP amidotransferase) (purF)	STM2362	0	0	2
343	serine hydroxymethyltransferase (glyA)	STM2555	4	5	3
344	putative glycosyl hydrolase	STM1559	2	3	5
345	putative periplasmic protein (ydgH)	STM1478	4	4	3
346	RNA polymerase, beta prime subunit (rpoC)	STM4154	0	7	3
347	involved in density-dependent regulation of (gcpE)	STM2523	5	0	6
348	NADH dehydrogenase I chain B (nuoB)	STM2327	4	4	4
349	3-phosphoserine aminotransferase (serC)	STM0977	4	3	5
350	NADH dehydrogenase I chain I (nuoI)	STM2321	3	4	4
351	outer membrane lipoprotein (lipocalin) (blc)	STM4339	2	4	3
352	malonyl-CoA-[acyl-carrier-protein] transacylase (fabD)	STM1194	3	3	2
353	component of clpA-clpP ATP-dependent serine protease (clpX)	STM0449	3	2	4
354	nucleoside diphosphate kinase (ndk)	STM2526	3	2	4
355	molecular chaperone in protein export (secB)	STM3701	2	2	2
356	Homolog of pipB, putative pentapeptide repeats	STM2780	3	3	2
357	putative ABC superfamily (atp_bind) transport protein (yhbG)	STM3319	1	3	2
358	putative uronate isomerase	STM3137	3	0	0
359	ABC superfamily (peri_perm), D-ribose transport protein (rbsB)	STM3884	5	2	5
360	dipeptidase for D-ala-D-ala digestion in peptidoglycan (pdgL)	STM1599	4	4	2
361	tryptophan tRNA synthetase (trpS)	STM3481	2	1	3
362	negative modulator of initiation of replication, inhibits (seqA)	STM0697	4	4	4
363	glucosamine-6-phosphate deaminase (nagB)	STM0684	2	3	4
364	GTPase domain of cell division membrane protein (ftsY)	STM3571	4	2	4
365	ABC superfamily (bind_prot), arginine transport system (artI)	STM0890	3	4	2
366	glycerolphosphate acyltransferase activity (plsB)	STM4235	1	4	5

367	thiol peroxidase, thioredoxin dependent (bcp)	STM2491	2	2	3
368	purine-binding chemotaxis protein regulation (cheW)	STM1920	2	3	2
369	putative cytoplasmic protein	STM1078	1	3	3
370	putative glycosyl hydrolase	STM1558	0	3	3
371	pyruvate kinase II, glucose stimulated (pykA)	STM1888	0	4	4
372	sigma S (sigma 38) factor of RNA (rpoS)	STM2924	4	0	2
373	proline dehydrogenase (putA)	STM1124	0	5	4
374	a late step of protoheme IX synthesis (hemY)	STM3935	3	2	3
375	galactokinase (galK)	STM0774	3	3	2
376	putative nitrate reductase	STM3377	2	4	4
377	chorismate pyruvate lyase (ubiC)	STM4233	2	4	4
378	Secreted effector protein of Salmonella dublin (sopA)	STM2066	3	4	4
379	transcription termination L factor (nusB)	STM0418	1	0	2
380	ABC superfamily (peri_perm), molybdate transporter (modA)	STM0781	4	3	3
381	oxaloacetate decarboxylase (eda)	STM1884	3	3	4
382	putative pectinesterase (ybhC)	STM0786	4	2	2
383	30S ribosomal subunit protein S8, and regulator (rpsH)	STM3426	2	3	2
384	transcriptional repressor of iron-responsive genes (Fur family) (fur)	STM0693	3	3	3
385	50S ribosomal subunit protein L20 (rplT)	STM1336	3	3	3
386	putative oxidoreductase / K + channel protein (yajO)	STM0421	2	3	2
387	outer membrane N-acetyl (apeE)	STM0570	5	3	2
388	periplasmic protein disulfide isomerase I (dsbA)	STM3997	3	2	3
389	putative cytoplasmic protein (ybeL)	STM0653	2	2	3
390	putative Homolog of glutamic dehyrogenase	STM1795	2	4	2
391	putative Phospholipid-binding protein (ybhB)	STM0792	2	3	3
392	ABC superfamily (peri_perm), putrescine transporter (potF)	STM0877	4	2	2
393	flavodoxin 1 (fldA)	STM0694	2	2	3
394	SAICAR synthetase (purC)	STM2487	2	3	3
395	DNA methylase M, host modification (hsdM)	STM4525	2	2	0
396	UDP-glucose pyrophosphorylase (galF)	STM2098	4	0	3
397	putative cellulose synthase (yhjN)	STM3618	3	1	4
398	putative cytoplasmic protein (ybgI)	STM0711	2	3	2
399	putative nucleic acid-binding protein, contains PIN domain	STM3033	0	0	2
400	putative ferripyochelin binding protein (yrdA)	STM3399	3	3	4
401	RNase E (rne)	STM1185	2	4	3
402	rod shape-determining protein HSP70 chaperones (mreB)	STM3374	3	2	3
403	N-acetylglucosamine-6-phosphate deacetylase (nagA)	STM0683	3	3	2
404	catalase hydroperoxidase HPI(I) (katG)	STM4106	2	4	4
405	ribosephosphate isomerase, constitutive (rpiA)	STM3063	3	2	5
406	4.5S-RNP protein, GTP binding export factor, part (ffh)	STM2677	2	3	5
407	peptidase component of the HslUV protease (hslV)	STM4092	3	2	3
408	putative outer membrane protein	STM1328	2	2	4
409	dehydroshikimate reductase (aroE)	STM3401	2	3	2
410	subunit of clpA-clpP ATP-dependent serine protease (clpP)	STM0448	2	2	2
411	putative Competence-damaged protein (ydeJ)	STM1514	2	2	2
412	putative peptide maturation protein, (pmbA)	STM4438	4	1	2
413	phosphoheptose isomerase (ghmA)	STM0310	0	3	2
414	transcriptional repressor of modABCD operon (modE)	STM0779	3	0	2
415	putative nucleotide binding (ygdH)	STM2969	4	0	3
416	transcriptional repressor for pur regulon, glyA, glnB (purR)	STM1430	2	0	0
417	putative inner membrane protein	STM4261	0	2	2
418	anaerobic dimethyl sulfoxide reductase, subunit A (dmsA)	STM0964	3	3	3
419	chemotactic response CheY protein phophatase (cheZ)	STM1915	3	2	2

420	transcriptional regulator of aromatic aminoacid biosynthesis (tyrR)	STM1683	3	2	0
421	threonine 3-dehydrogenase (tdh)	STM3708	2	3	3
422	putative GTP-binding protein (yhbZ)	STM3301	3	3	3
423	periplasmic murein tripeptide transport protein (mppA)	STM1679	2	3	3
424	putative formate acetyltransferase (yfiD)	STM2646	2	2	3
425	glucose-1-phosphate uridylyltransferase (galU)	STM1752	2	2	2
426	putative cytoplasmic protein (samB)	PSLT051	2	3	4
427	FKBP-type peptidyl-prolyl cis-trans isomerase (rotamase) (fklB)	STM4397	3	2	3
428	putative cytoplasmic protein	STM1324	3	3	2
429	putative oxidoreductase (yigC)	STM3978	2	3	3
430	putative transaldolase (talC)	STM4109	2	3	2
431	3-ketoacyl-CoA thiolase (thiolase I, acetyl-CoA transferase) (fadA)	STM3982	2	2	3
432	beta-hydroxydecanoyl thioester dehydrase (fabA)	STM1067	2	2	2
433	putative integrase protein	PSLT042	2	3	2
434	believed to be involved in assembly of (yfhP)	STM2544	2	3	2
435	putative lipoprotein (ybjP)	STM0892	3	1	2
436	CDP glucose 4,6-dehydratase (rfbG)	STM2091	0	4	2
437	putative oxoacyl-(acyl carrier protein) reductase (yciK)	STM1717	4	0	3
438	lipoprotein, cell division (nlpI)	STM3281	2	0	1
439	D-ribulose-5-phosphate 3-epimerase (rpe)	STM3483	3	2	0
440	putative ABC transporter periplasmic binding protein (yliB)	STM0849	3	0	5
441	50S ribosomal subunit protein L21 (rplU)	STM3304	0	3	3
442	aspartate tRNA synthetase (aspS)	STM1901	0	4	3
443	2:3-cyclic-nucleotide 2-phosphodiesterase (cpdB)	STM4403	0	3	2
444	surface presentation of antigens secretory proteins (spaO)	STM2891	0	2	2
445	putative acetyltransferase	STM2449	2	2	1
446	RNase I, cleaves phosphodiester bond between any (rna)	STM0617	2	2	0
447	D-alanyl-D-alanine carboxypeptidase (dacA)	STM0637	3	3	2
448	exonuclease III, may repair singlet oxygen induced (xthA)	STM1302	1	2	1
449	putative inner membrane protein (yfgM)	STM2521	3	0	2
450	3-deoxy-D-arabinoheptulosonate-7-phosphate synthase (aroG)	STM0760	2	3	3
451	paral putative membrane protein (yfgA)	STM2524	2	3	3
452	methyl-accepting transmembrane (tcp)	STM3577	2	3	3
453	response regulator in two-component regulatory system (cpxR)	STM4059	3	2	3
454	putative bacterial regulatory protein, merR family (yjdC)	STM4322	3	3	2
455	putative transcriptional regulator (ytfJ)	STM4405	3	2	3
456	preprotein translocase secretion protein of IISP family (secA)	STM0136	2	2	3
457	SLP2_0011 undefined product 6669:8015 forward MW:51227	SLP2_0011	2	2	3
458	putative cytoplasmic protein (yjbQ)	STM4250	3	1	2
459	putative cytoplasmic protein (yggL)	STM3108	2	2	2
460	stringent starvation protein B (sspB)	STM3341	2	2	2
461	ABC superfamily (binding protein), vitamin B12 transport (btuE)	STM1341	2	2	0
462	L-glutamine:D-fructose-6-phosphate aminotransferase (glmS)	STM3861	0	0	3
463	SOS response regulator, (LexA family) (lexA)	STM4237	2	0	2
464	putative translation factor (yciO)	STM1720	2	0	2
465	putative ATPase involved in chromosome partitioning (yhjQ)	STM3620	0	0	5
466	6-phosphofructokinase I (pfkA)	STM4062	0	2	2
467	histidine tRNA synthetase (hisS)	STM2522	0	2	3
468	glutamate-1-semialdehyde aminotransferase (hemL)	STM0202	3	0	4
469	glutamine tRNA synthetase (glnS)	STM0686	0	3	2
470	putative DegT/DnrJ/EryC1/StrS family (yfbE)	STM2297	3	3	0
471	a minor lipoprotein (rlpA)	STM0638	5	2	0
472	uroporphyrinogen III methylase (hemX)	STM3936	4	0	4
473	invasion genes transcription activator (hilA)	STM2876	0	3	4

474	putative enzyme with a TIM-barrel fold (yggS)	STM3100	3	0	3
475	DNA strand exchange and recombination protein with (recA)	STM2829	0	4	2
476	Propanediol utilization: polyhedral bodies (pudB)	STM2039	0	2	3
477	putative S-adenosyl methionine adenyltransferase (yabC)	STM0120	0	2	3
478	putative periplasmic protein (ydeI)	STM1515	3	0	0
479	putative methyl-accepting chemotaxis protein	STM3152	0	3	0
480	a minor lipoprotein (rlpB)	STM0647	3	2	2
481	putative periplasmic protein (ybiS)	STM0837	2	3	2
482	putative chromosome partitioning (mukE)	STM0993	3	2	2
483	curved DNA-binding protein (cbpA)	STM1112	2	3	2
484	integration host factor (IHF), alpha subunit (himA)	STM1339	2	2	3
485	trehalose-6-phosphate phophatase, biosynthetic (otsB)	STM1929	3	2	2
486	putative xylanase/chitin deacetylase	STM3132	3	2	2
487	putative glutathione S-transferase (yghU)	STM3140	2	3	2
488	putative phosphosugar isomerase	STM3601	2	2	3
489	branched-chain amino-acid aminotransferase (ilvE)	STM3903	3	2	2
490	putative alcohol dehydrogenase (yjgB)	STM4486	3	2	2
491	putative secreted protein (yceI)	STM1157	2	2	2
492	riboflavin synthase, alpha chain (ribE)	STM1426	2	2	2
493	peptidyl-tRNA hydrolase (pth)	STM1783	1	2	3
494	succinylarginine dihydrolase (astB)	STM1306	2	2	2
495	peptide deformylase (def)	STM3406	2	2	1
496	N-acetylneuraminate lyase (aldolase) (nanA)	STM3339	2	2	1
497	putative tRNA/rRNA methyltransferase (yfiF)	STM2648	2	1	2
498	1,4-alpha-glucan branching enzyme (glgB)	STM3538	2	0	0
499	30S ribosomal subunit protein S11 (rpsK)	STM3417	3	3	0
500	thymidine kinase (tdk)	STM1750	0	3	3
501	Secretion system effector (sseC)	STM1400	0	4	0
502	putative Thioredoxin-like proteins and domain (yhgI)	STM3511	1	0	3
503	putative ATPase involved in cell division (ftsE)	STM3570	2	0	4
504	ABC superfamily glycine/betaine/proline transport protein (proX)	STM2811	2	2	0
505	inositol monophosphatase (suhB)	STM2546	1	2	0
506	three acitivities: regulator of nadAB transcription, regulator (nadR)	STM4580	0	3	2
507	unknown function in glycerol metabolism (glpX)	STM4085	0	2	2
508	transcriptional activator of ntrL gene (osmE)	STM1311	2	0	0
509	glycine cleavage complex protein T, (gcvT)	STM3055	2	3	0
510	aerotaxis sensor receptor, senses cellular redox state (aer)	STM3217	2	3	0
511	SLP1_0054 undefined product 47844:48821 reverse MW:36820	SLP1_0054	0	0	4
512	glutamine synthetase (glnA)	STM4007	0	0	4
513	glycogen phosphorylase (glgP)	STM3534	0	0	3
514	outer membrane lipoprotein (lolB)	STM1778	3	0	4
515	heat shock protein 33, redox regulated chaparone (yrfI)	STM3498	0	2	2
516	sugar specific PTS family, enzyme IIA, also (ptsN)	STM3322	4	0	0
517	2-oxo-3-deoxygalactonate 6-phosphate aldolase (dgoA)	STM3828	6	0	0
518	SLP2_0012 undefined product 8294:10174 forward MW:70003	SLP2_0012	0	4	0
519	ABC superfamily lysine/arginine/ornithine transport protein (argT)	STM2355	4	0	0
520	glutamine amidotransferase, subunit with HisF (hisH)	STM2075	2	0	0
521	4-methyl-5(beta-hydroxyethyl)-thiazole synthesis (thiJ)	STM0433	2	2	2
522	lipoprotein (nlpD)	STM2925	2	2	2
523	small heat shock protein (ibpA)	STM3809	2	2	2
524	SLP2_0010 undefined product 5713:6315 reverse MW:22464	SLP2_0010	1	2	2
525	phenylalanine tRNA synthetase, alpha-subunit (pheS)	STM1337	2	1	2

526	non-specific acid phosphatase (phoN)	STM4319	2	1	2
527	paral putative methyltransferase tellurite resistance (tehB)	STM1608	0	0	3
528	putative P-loop-containing kinase (yhbJ)	STM3323	2	2	0
529	putative negative regulator (yfeD)	STM2414	1	1	1
530	aspartate-semialdehyde dehydrogenase (asd)	STM3539	1	0	3
531	putative oxidoreductase (ydgJ)	STM1462	0	0	4
532	putative sarcosine oxidase (solA)	STM1160	0	0	2
533	Secretion system effector (sseA)	STM1397	0	2	2
534	response regulator in two-component regulatory system (narP)	STM2246	0	2	3
535	protoporphyrin oxidase (hemG)	STM3987	0	2	2
536	ssDNA-binding protein controls activity of RecBCD nuclease (ssb)	STM4256	0	2	2
537	shikimate kinase I (aroK)	STM3487	0	2	1
538	Homolog of slsA in STM	STM0950	2	0	3
539	response regulator in two-component regulatory system with	STM4292	2	0	3
540	sigma cross-reacting protein 27A (SCRP-27A)	STM3327	0	2	3
541	methionine adenosyltransferase 1 (AdoMet synthetase) (metK)	STM3090	0	3	2
542	glutathione oxidoreductase (gor)	STM3597	2	0	3
543	periplasmic nitrate reductase, large subunit, in complex (napA)	STM2259	0	2	3
544	proline dipeptidase (pepQ)	STM3984	0	0	3
545	putative citrate synthase (prpC)	STM0369	3	2	0
546	threonine synthase (thrC)	STM0004	2	2	0
547	acetylornithine deacetylase (argE)	STM4120	0	0	2
548	outer membrane protein receptor / transporter for (fhuA)	STM0191	3	0	3
549	putative alpha amylase	STM1560	0	3	3
550	DNA topoisomerase type I, omega protein (topA)	STM1714	0	3	3
551	putative enzyme (yniC)	STM1322	2	0	3
552	outer membrane receptor for transport of vitamin (btuB)	STM4130	2	0	3
553	cell invasion protein (prgH)	STM2874	0	3	2
554	glucokinase (glk)	STM2403	0	3	2
555	activator of proP (proQ)	STM1846	1	0	2
556	putative flagellar biosynthesis(orgA)	STM2869	2	0	2
557	sensory histidine kinase (barA)	STM2958	0	0	3
558	surface presentation of antigens secretory proteins (invJ)	STM2892	0	3	0
559	putative enzymes related to aldose 1-epimerase (yeaD)	STM1289	0	0	3
560	putative alcohol dehydrogenase (yqhD)	STM3164	0	0	2
561	putative glutathione S-transferase	STM4267	0	0	3
562	putative outer membrane lipoprotein (ycfM)	STM1207	0	2	0
563	putative cytoplasmic protein (yceH)	STM1168	0	0	1
564	flagellar biosynthesis, hook protein (flgE)	STM1177	1	0	0
565	ribosome-binding factor, role in processing of 10S (rbfA)	STM3285	1	2	0
566	putative Rhodanese-related sulfurtransferases (yibN)	STM3703	2	0	2
567	ecotin, a serine protease inhibitor (eco)	STM2262	0	2	2
568	adenine phosphoribosyltransferase (apt)	STM0483	0	2	2
569	3,4 dihydroxy-2-butanone-4-phosphate synthase (ribB)	STM3195	2	2	0
570	endonuclease V (deoxyinosine 3endoduclease) (nfi)	STM4168	2	0	0
571	putative inhibitor of septum formation (yceF)	STM1189	0	2	3
572	pantothenate synthetase (panC)	STM0181	0	1	0
573	tartronate semialdehyde reductase (TSAR) (garR)	STM3248	2	0	0
574	peptide chain release factor RF-2 (prfB)	STM3041	0	2	2
575	proline aminopeptidase P II (pepP)	STM3058	0	2	2
576	phosphoglucosamine mutase (mrsA)	STM3294	0	2	2
577	UDP-N-acetylglucosamine 1-carboxyvinyltransferase (murA)	STM3307	0	2	2
578	putative helicase (rhlB)	STM3914	0	2	2

579	phosphate starvation-inducible protein, ATP-binding (phoL)	STM0669	2	0	2
580	carboxy-terminal protease for penicillin-binding protein 3 (prc)	STM1845	0	3	2
581	transcriptional regulator for hemolysin (MarR family) (slyA)	STM1444	0	2	0
582	dihydroxyacid dehydratase (ilvD)	STM3904	2	0	2
583	uridine phosphorylase (udp)	STM3968	0	3	0
584	cell invasion protein lipoprotein, may link inner (prgK)	STM2871	0	2	0
585	acetylCoA carboxylase, beta subunit (accD)	STM2366	3	0	0
586	cytidine monophosphate (CMP) kinase (cmk)	STM0980	2	2	0
587	erythronate-4-phosphate dehyrogenase (pdxB)	STM2370	0	0	3
588	putative cytoplasmic protein (ycfD)	STM1229	0	0	2
589	Gifsy-2 prophage	STM1031	0	0	2
590	cysteine tRNA synthetase (cysS)	STM0537	2	0	3
591	regulator of length of O-antigen component of (wzzB)	STM2079	3	2	0
592	lipoprotein precursor (vacJ)	STM2392	0	2	3
593	dTDP-4,deoxyrhamnose 3,5 epimerase (rfbC)	STM2094	2	2	0
594	putative LysM domain (ynhG)	STM1375	2	0	2
595	(alpha)-aspartyl dipeptidase (pepE)	STM4190	0	2	2
596	30S ribosomal subunit protein S10 (rpsJ)	STM3441	0	1	1
597	putative oxidoreductase (yohF)	STM2171	0	2	1
598	guanylate kinase (gmk)	STM3740	0	1	0
599	3-oxoacyl-[acyl-carrier-protein] synthase II (fabF)	STM1197	1	0	2
600	putative mannonate hydrolase	STM3135	0	0	2
601	adenylosuccinate lyase (purB)	STM1232	0	2	0
602	TypeIII-secreted protein effector (sopE2)	STM1855	0	0	2
603	thymidine phosphorylase (deoA)	STM4568	0	0	2
604	membrane-bound ATP synthase, F1 sector, delta-subunit (atpH)	STM3868	0	3	0
605	histone-like protein, located in outer membrane (hlpA)	STM0225	2	0	0
606	nucleotide associated protein(yejK)	STM2226	2	0	0
607	NifU homologs involved in Fe-S cluster formation (nifU)	STM2542	1	0	0
608	cytoplasmic ferritin (ftn)	STM1935	0	2	0
609	deoxyuridinetriphosphatase (dut)	STM3731	0	2	0
610	flagellar biosynthesis (fliL)	STM1975	0	1	0
611	putative arsenate reductase (yfgD)	STM2495	0	0	1
612	transcription elongation factor, cleaves 3 nucleotide of (greA)	STM3299	0	0	2
613	putative membrane protein, involved in stability of (yggB)	STM3067	0	0	2
614	Fels-1 prophage putative minor tail protein	STM0918	0	2	0
615	cob(I)alamin and cobinamide adenolsyltransferase (btuR)	STM1718	0	2	0
616	putative inner membrane protein	STM2870	0	2	0
617	oligoribonuclease (orn)	STM4350	0	2	0
618	peptide methionine sulfoxide reductase (msrA)	STM4408	0	2	0
619	dnaK suppressor protein (dksA)	STM0186	0	1	0
620	putative RHS-family protein	STM0291	2	0	0
621	putative Xanthosine triphosphate pyrophosphatase (yggV)	STM3103	2	0	0
622	paral putative enzyme (yihX)	STM4026	2	0	0
623	putative methyltransferase (menG)	STM4089	2	0	0
624	ferrochelatase (hemH)	STM0489	0	2	0
625	3-oxoacyl-[acyl-carrier-protein] synthase III acetylCoA (fabH)	STM1193	0	2	0
626	glycerol-3-phosphate dehydrogenase (NAD+) (gpsA)	STM3700	0	1	0
627	putative outer membrane or secreted lipoprotein	STM1561	2	0	0
628	flagellar biosynthesis, component of motor switching and (fliG)	STM1970	2	0	0
629	porphobilinogen deaminase (hemC)	STM3938	2	0	0
630	glutathione synthetase (gshB)	STM3095	0	0	2
631	putative sensor/kinase in regulatory system (yojN)	STM2269	2	0	0

632	paral putative outer membrane receptor (yncD)	STM1587	0	0	2
633	putative inner membrane protein	STM2208	0	2	0
634	Alanyl-alanine carboxypeptidase penicillin-binding protein (dacC)	STM0863	0	2	0
635	N-acetyl glucosamine-1-phosphate uridyltransferase (glmU)	STM3862	0	2	0
636	sensory kinase in two-component regulatory system with (cpxA)	STM4058	3	0	0
637	UDP-N-acetyl-muramate:alanine ligase (murC)	STM0129	2	0	0
638	gamma-glutamate-cysteine ligase (gshA)	STM2818	2	0	0
639	succinylglutamic semialdehyde dehydrogenase (astD)	STM1305	2	2	0
640	cell division topological specificity factor, reverses MinC (minE)	STM1816	0	2	2
641	hydrogenase-2, large subunit (hybC)	STM3147	0	2	2
642	RNase PH (rph)	STM3734	2	2	0
643	small heat shock protein (ibpB)	STM3808	0	2	2
644	putative GTP-ase, together with HflCK possibly involved (hflX)	STM4362	2	0	2
645	UvrA with UvrBC is a DNA excision (uvrA)	STM4254	1	0	2
646	putative glycosyl transferase (pmrF)	STM2298	0	2	1
647	putative outer membrane lipoprotein (yceB)	STM1164	1	0	2
648	putative cytoplasmic protein (yqiC)	STM3196	1	0	2
649	SLP2_0021 undefined product 15468:16439 forward MW:35713	SLP2_0021	0	0	2
650	putative SH3 domain protein (ygiM)	STM3203	0	2	0
651	succinate dehydrogenase, cytochrome b556 (sdhC)	STM0732	2	0	0
652	Secretion system chaparone (sscA)	STM1399	0	1	0
653	non-specific acid phosphatase/phosphotransferase, class B (aphA)	STM4249	0	0	1
654	alpha-amylase (malS)	STM3664	0	0	1
655	dihydropteridine reductase (nfnB)	STM0578	0	0	3
656	putative dienelactone hydrolase family (dlhH)	STM3967	1	0	0
657	anti sigma E (sigma 24) factor, negative (rseB)	STM2638	0	1	0
658	phosphoenolpyruvate carboxylase (ppc)	STM4119	0	3	0
659	UDP-sugar hydrolase 5-nucleotidase (ushA)	STM0494	0	0	2
660	30S ribosomal subunit protein S14 (rpsN)	STM3427	0	1	0
661	paral putative transformylase (yfbG)	STM2299	0	1	0
662	putative Fe-S-cluster redox enzyme (yfgB)	STM2525	0	0	3
663	putative transcriptional regulator (ybaD)	STM0415	0	0	2
664	Gifsy-2 prophage probable regulatory protein	STM1012	0	0	2
665	putative MutT-like protein (ymfB)	STM1235	0	2	0
666	putative TPR-repeat-containing protein (yhjL)	STM3616	2	0	0
667	putative carboxylase (ybgJ)	STM0712	0	0	2
668	putative phosphatase (yfbT)	STM2334	0	0	2
669	dUTPase (dcd)	STM2121	0	0	2
670	putative periplasmic protein (yggN)	STM3107	0	0	2
671	putative phosphoesterase (yfcE)	STM2347	0	2	0
672	UDP-N-acetylglucosamine acetyltransferase (lpxA)	STM0228	0	2	0
673	putative sugar nucleotide epimerase (yfcH)	STM2350	2	0	0
674	dihydroxynaphtoic acid synthetase (menB)	STM2307	0	2	0
675	cell invasion protein (sipD)	STM2883	0	2	0
676	dTDP-glucose pyrophosphorylase (rfbA)	STM2095	0	1	0
677	glycine tRNA synthetase, alpha subunit (glyQ)	STM3656	0	0	2
678	SLP2_0044 undefined product 30311:31360 forward MW:39469	SLP2_0044	0	0	1
679	putative purine nucleoside hydrolase (ybeK)	STM0661	1	0	0
680	outer membrane phospholipase A (pldA)	STM3957	2	0	0
681	putative hydrolase of the alpha/beta superfamily (yafA)	STM0318	0	0	2
682	SLP1_0055 undefined product 48818:50023 reverse MW:44501	SLP1_0055	0	0	2
683	ATP-binding subunit of serine protease (clpA)	STM0945	0	0	2
684	DNA polymerase III, beta-subunit (dnaN)	STM3837	0	0	2

685	fermentative D-lactate dehydrogenase, NAD-dependent (ldhA)	STM1647	0	3	0
686	putative outer membrane or exported	STM4242	3	0	0
687	putative translation initiation inhibitor (yjgF)	STM4458	0	3	0
688	SLP2_0065 undefined product 54440:55144 reverse MW:26137	SLP2_0065	0	1	0
689	dehydroquinate synthase (aroB)	STM3486	0	0	2
690	putative transcriptional regulator (yqgE)	STM3096	2	0	0
691	putative cytoplasmic protein (yaeQ)	STM0239	0	0	2
692	pyruvate dehydrogenase/oxidase (poxB)	STM0935	0	2	0
693	RNase T, degrades tRNA, has exonuclease and (rnt)	STM1434	2	0	0
694	putative hydrogenase-1 large subunit	STM1538	0	2	0
695	N-methylation of lysine residues in flagellin (fliB)	STM1958	0	0	2
696	flagellar biosynthesis basal-body MS-ring and (fliF)	STM1969	2	0	0
697	putative hydrolase of the HAD superfamily (yedP)	STM1986	0	0	2
698	putataive glutathione-S-transferase (yfcF)	STM2348	0	0	2
699	ABC superfamily (bind_prot), thiosulfate transport protein (cysP)	STM2444	0	0	2
700	sigma E (sigma 24) factor of (rpoE)	STM2640	0	0	2
701	16S rRNA processing protein (rimM)	STM2675	2	0	0
702	General PTS system, enzyme I, transcriptional regulator (ptsP)	STM3003	0	0	2
703	putative D-mannonate oxidoreductase	STM3136	2	0	0
704	putative periplasmic protein (yqjC)	STM3228	2	0	0
705	putative DNA topoisomerase (yrdD)	STM3403	0	2	0
706	putative oxidoreductase (yieF)	STM3850	0	0	2
707	homocysteine-N5-methyltetrahydrofolate transmethylase (metH)	STM4188	0	0	2
708	putative phosphoglyceromutase 2 (gpmB)	STM4585	0	0	2
709	putative ABC superfamily (membrane) transport protein (ybhR)	STM0815	0	0	1
710	putative envelope protein (envE)	STM1242	0	0	1
711	host factor I for bacteriophage Q beta (hfq)	STM4361	1	0	0
712	putative ABC transporter periplasmic binding protein	STM1255	0	1	0
713	phosphatidylserine decarboxylase (psd)	STM4348	0	1	0

Chapter 4

AN RPOS-DEPENDENT SRNA REGULATES THE EXPRESSION OF A CHAPERONE INVOLVED IN PROTEIN FOLDING

This chapter contains data that was submitted in the article:

Silva, I.J., Ortega, A.D.*, Viegas, S.C.*, Portillo, F.G., and Arraiano, C.M. 2012. An RpoS-dependent sRNA regulates the expression of a chaperone involved in protein folding. **Accepted with modifications in RNA Journal**

*These authors contributed equally to this work.

The author of this Dissertation is the first author of the submitted manuscript.

The proteomic experiments and the majority of the studies concerning RpoS were performed by Professor Francisco García-del Portillo and Dr. Álvaro Darío Ortega from Departamento de Biotecnología Microbiana, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas (CNB-CSIC), Madrid, Spain.

ABSTRACT

Small non-coding RNAs (sRNAs) are usually expressed in the cell to face a variety of stresses. In this report we disclose the first target for SraL (also known as RyjA), a sRNA present in many bacteria, which is highly induced in stationary phase. We also demonstrate that this sRNA is directly transcribed by the major stress sigma factor σ^S (RpoS) in *Salmonella enterica* serovar Typhimurium. We show that SraL sRNA down-regulates the expression of the chaperone Trigger Factor (TF), encoded by the *tig* gene. TF is one of the three major chaperones which cooperate in the folding of the newly synthesized cytosolic proteins. Trigger factor is the only ribosome associated chaperone known in bacteria. By using bioinformatic tools and mutagenesis experiments, SraL was shown to interact with the 5'-UTR of the *tig* mRNA few nucleotides upstream of the Shine-Dalgarno region. This work constitutes the first report of a small RNA affecting protein folding. Taking into account that both SraL and TF are very well conserved in enterobacteria this work will have important repercussions in the field.

INTRODUCTION

Small non-coding RNAs (sRNAs) perform a wide diversity of regulatory functions in both prokaryotic and eukaryotic cells. The majority of the sRNAs act by base pairing with mRNA targets (antisense sRNAs) or by binding to proteins to modify their activity (for a review see (Storz *et al.*, 2011)). Most of the antisense sRNAs are *trans*-encoded since they are encoded in a separate locus in relation with the mRNA target. Consequently, these sRNAs exhibit only partial complementarity with the target and usually require the RNA chaperone Hfq for base pairing. Typically, *trans*-encoded sRNAs are induced under environmental stress conditions and also upon entry into stationary phase of growth in order to up- or down-regulate their target(s) (Gottesman and Storz, 2011).

A plethora of sRNAs has been identified in the last years; for instance in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) 140 sRNAs were reported in early stationary phase of growth by using a combination of RNA-seq and dRNA-seq analysis and Hfq-coIP-seq approach (Kröger *et al.*, 2012).

sRNAs are generally highly controlled at the transcriptional level. Nearly one-third of the functional characterized sRNAs contribute to the control of the outer membrane protein production. Part of these sRNAs is under the control of the sigma factor RpoE (also known as σ^E or σ^{24}) (Johansen *et al.*, 2008; Johansen *et al.*, 2006; Papenfort *et al.*, 2006; Udekwu and Wagner, 2007), which regulates gene expression upon the accumulation of misfolded outer membrane proteins (OMPs) in the periplasmic space (Mecsas *et al.*, 1993; Missiakas *et al.*, 1996; Raivio and Silhavy, 1999). However, only a few sRNAs have been reported to be transcribed by the sigma factor RpoS (also known as σ^S or σ^{38}) (Fröhlich *et al.*, 2012; Opdyke *et al.*, 2004; Padalon-Brauch *et al.*, 2008). This major stress sigma factor regulates 10% of the *E. coli* genes (Weber *et al.*, 2005) and is induced under several stress

conditions namely the entry in stationary phase of growth (Battesti *et al.*, 2011). RpoS is known to play important roles in the virulence of many bacterial pathogens, including *S*. Typhimurium (Dong and Schellhorn, 2010).

SraL (also known as RyjA) is a 140-nucleotides antisense sRNA firstly described in 2001 in two genome-wide searches for new sRNAs (Argaman *et al.*, 2001; Wassarman *et al.*, 2001). More recently, this sRNA was also detected in *S.* Typhimurium (Ortega *et al.*, 2012; Viegas *et al.*, 2007). SraL sRNA was only detected in cells upon entry into stationary phase (Argaman *et al.*, 2001; Viegas *et al.*, 2007; Wassarman *et al.*, 2001), and its expression is particularly high in late stationary phase. Moreover, its expression was also highly detected under *Salmonella*-pathogenicity island-2 (SPI-2) inducing conditions (Viegas *et al.*, 2007), which indicates a possible role for SraL in *Salmonella* virulence since SPI-2 genes are important for intra-macrophage survival and systemic disease. SraL is also expressed in intracellular *S.* Typhimurium persisting inside eukaryotic cells (Ortega *et al.*, 2012). The study of the post-transcriptional regulation of SraL through the use of several *Salmonella* ribonucleases mutants showed that this sRNA is controlled by RNases such as PNPase and the degradosome complex (Viegas *et al.*, 2007) (Figure 1A). Moreover, there was also an accumulation of a smear of slightly larger transcripts (most likely polyadenylated precursors) in both mutants (indicated as X in Figure 1A). Additionally, it was shown that PAP I has a major impact in the control of the stability of this sRNA and the bands corresponding to longer SraL molecules were absent, supporting that SraL is polyadenylated (Viegas *et al.*, 2007) (Figure 1B). This fact was in agreement with previous 3′ RACE experiments that revealed the existence of 3′ A-tails of different lengths in the *E. coli* SraL transcript (Argaman *et al.*, 2001). The absence of RNase III caused the accumulation of the larger band (indicated as X in Figure 1B).

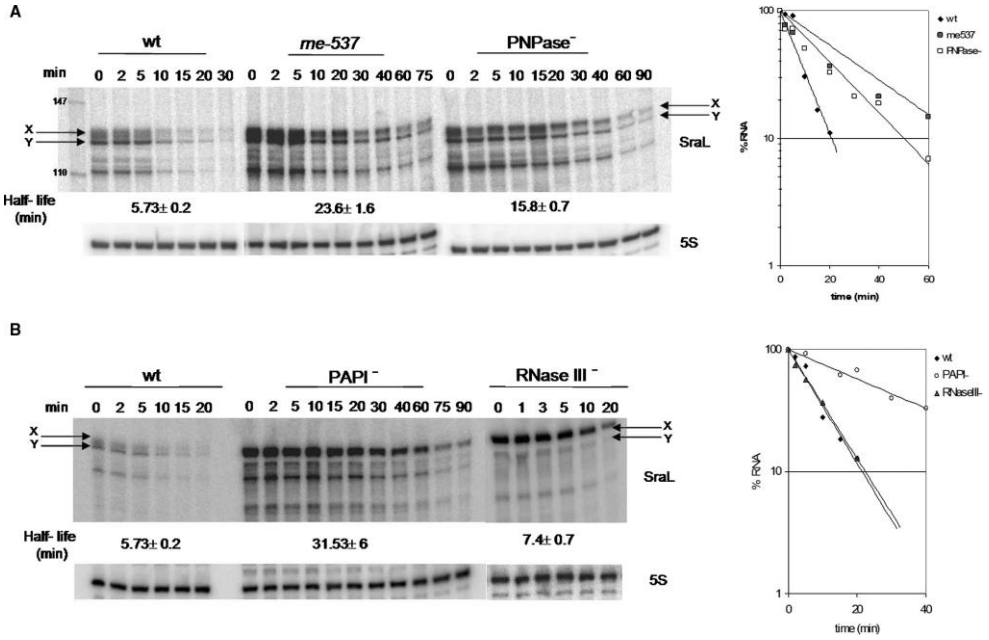

FIGURE 1 – Analysis of the role of RNase E, PNPase, RNase III and PAP I in SraL regulation. **(A)** Comparison of *rne-537* and PNPase mutations in SraL decay. **(B)** Analysis of the effect of RNase III and PAP I in the decay and processing of SraL transcript. Adapted from (Viegas *et al.*, 2007).

In this work, we have determined that RpoS (the major stationary phase regulator) is a transcriptional regulator of the highly conserved sRNA SraL in *S.* Typhimurium. SraL transcription is dependent on the presence of RpoS in the cell and we have proved that this regulation is direct since RpoS directly binds to the promoter of the *sraL* gene. We have also investigated the biological role of SraL since no targets were yet discovered for this sRNA. A proteomic analysis using a *S*. Typhimurium SraL null mutant and a SraL overexpressing strain detected Trigger Factor (TF) as a possible target. TF is one of the three major cytosolic chaperone proteins found in all eubacteria and assists in protein folding (Hesterkamp *et al.*, 1996). This chaperone is the only ribosome associated chaperone known in bacteria (Hoffmann *et al.*, 2010). By using mutational

analysis, we have determined that SraL represses *tig* mRNA through a short stretch of complementarity in the *tig* 5'-UTR near the Shine-Dalgarno region. The results obtained in this study constitute the first link between sRNAs and protein folding.

EXPERIMENTAL PROCEDURES

Oligonucleotides

All oligonucleotides used in this study are listed in the Supplementary Table S1 in the "Supplementary Information" section, and were synthesized by STAB Vida and Sigma-Aldrich.

Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are listed in the Table 1 and Table 2, respectively. All *Salmonella* strains used are isogenic derivates of the wild-type *Salmonella enterica* serovar Typhimurium strain SL1344. The *sraL* (CMA-651) and *tig* (CMA-652) null mutants were constructed using the primer pairs pIS-001/pIS-002 and pIS-005/pIS-006, respectively, and following the *λ-red* recombinase method (Datsenko and Wanner, 2000) with few modifications, as previously described (Viegas *et al.*, 2007). All chromosomal mutations were subsequently transferred to a fresh genetic background (SL1344 strain) by P22 HT105/1 int-201 transduction (Schmieger, 1971). The chloramphenicol-resistance cassette of plasmid pKD3 replaces nucleotides -9 to +120 of the *sraL* gene and -16 to +1303 of *tig*. The gene deletions were verified by colony PCR using the primer pair pIS-003/pIS-004 for *sraL* and pIS-007/pIS-008 for *tig*. The *S.* Typhimurium *rpoS* null mutant (CMA-653) was obtained by P22 transduction from SV4210 strain (Tierrez and Garcia-del Portillo, 2004).

For construction of pISVA-01 plasmid expressing SraL, a PCR fragment containing the entire *sraL* sequence was amplified from SL1344 chromosome using the primer pair pIS-009/pIS-010. The resultant PCR fragment carrying a 5′-phosphate at one end was cleaved with KpnI and ligated into the constitutive pZE12luc plasmid (blunt/KpnI site) (Lutz and Bujard, 1997). Plasmid expressing the mutated version of SraL (pISVA-02) was constructed using the same strategy but with the primer pair pIS-010/pIS-011. In these plasmids, the initiation site of the encoded RNA lies at position +1 of the constitutive PLlacO promoter of pZE12luc plasmid.

For the *rpoS* complementation plasmid pISVA-03, a PCR fragment containing the entire *rpoS* sequence was amplified from SL1344 chromosome using the primer pair pIS-013/pIS-014 and was cloned into the XbaI and HindIII sites of the plasmid pWSK29 (Wang and Kushner, 1991).

For the construction of plasmid pISVA-004 (P_{SraL}::*lacZ*), a fragment of the 5′-UTR region of SraL gene including promoter signals was amplified by PCR with primers pIS-014 and pIS-015 (containing the restriction sites for XbaI and BamHI, respectively). Both the insert and pSP417 vector were digested with XbaI and BamHI enzymes and ligated.

Competent *E. coli* DH5α cells (*New English Biolabs*) were used for cloning procedures during plasmid construction.

TABLE 1 - List of strains used in this work

Strain	Relevant Markers / Genotype	Source/Reference
S. Typhimurium, SL1344	Str^R *hisG rpsL xyl*	(Hoiseth and Stocker, 1981)
CMA-651	SL1344 *sraL* (Δ*sraL*::Cm^R)	This study
CMA-652	SL1344 *tig* (Δ*tig*::Cm^R)	This study
CMA-653	SL1344 *rpoS* (Δ*rpoS*::Cm^R)	This study
E. coli DH5α	*rec*A1 *end*A1 *gyr*A96 thi-*hsd*R17 *sup*E44 *rel*A1 Δ*lac*ZYA-*arg* FU169 f80dLacZDM15	*New England Biolabs*

TABLE 2 - List of plasmids used in this work

Plasmid	Comments	Origin/Marker	Source/Reference
pKD3	Template for mutants construction; carries chloramphenicol-resistance cassete	oriRγ/Amp^R	(Datsenko and Wanner, 2000)
pKD46	Temperature-sensitive λ-red recombinase expression plasmid	oriR101/Amp^R	(Datsenko and Wanner, 2000)
pZE12Luc	P$_{LlacO}$ promoter; constitutive expression plasmid	ColE1/Amp^R	(Lutz and Bujard, 1997)
pWSK29	Constitutive expression plasmid	pSC101/Amp^R	(Wang and Kushner, 1991)
pSP417	*lacZ* transcriptional fusion vector	pBR322/Amp^R	(Podkovyrov and Larson, 1995)
pISVA-001	pZE12luc derivative; P$_{LlacO}$ promoter; constitutive plasmid expressing SraL	ColE1/Amp^R	This study
pISVA-002	pZE12luc derivative; P$_{LlacO}$ promoter constitutive plasmid expressing the mutated version of SraL sRNA (SraL*)	ColE1/Amp^R	This study
pISVA-003	pWSK29 derivative; constitutive expression plasmid RpoS	pSC101/Amp^R	This study
pISVA-004	Transcriptional *sraL-lacZ* fusion	pBR322/Amp^R	This study

Bacterial growth

All strains were grown in LB broth at 37ºC and 220 r.p.m. throughout this study. SOC medium was used to recover transformants after heat shock (in the case of *E. coli*) or electroporation (in the case of *Salmonella*), before plating.

Growth medium was supplemented with the following antibiotics when appropriate: ampicillin (150 μg/ml), chloramphenicol (25 μg/ml) and streptomycin (90 μg/ml). To apply osmotic shock, cells were grown at 37ºC to an OD_{600} of 0.3. NaCl was added to the culture at a final concentration of 0.5 M.

RNA extraction, Northern blot and Reverse Transcription-PCR (RT-PCR) analysis

Overnight cultures were diluted 1/100 in fresh medium and grown to the indicated cell densities at OD_{600} (growth medium and conditions are detailed in the respective figure legends). Culture samples were collected, mixed with 1 volume of stop solution (10 mM Tris pH 7.2, 25 mM $NaNO_3$, 5 mM $MgCl_2$, 500 μg/ml chloramphenicol), and harvested by centrifugation (10 min, 6000 g, 4ºC). RNA was isolated using the phenol/chlorophorm extraction method, precipitated in ethanol, resuspended in water and quantified on a Nanodrop 1000 machine (*NanoDrop Technologies*).

For Northern blot analysis, 15 μg of total RNA was separated under denaturing conditions either by 8.3 M urea / 6% polyacrylamide gel in TBE buffer or by 1.3% agarose MOPS / formaldehyde gel. For polyacrylamide gels, transfer of RNA onto Hybond-N^+ membranes (*GE Healthcare*) was performed by electroblotting (1 h 50 min, 24 V, 4ºC) in TAE buffer. For agarose gels, RNA was transferred to Hybond-N^+ membranes by capillarity using 20x SSC as transfer buffer. In both cases, RNA was UV crosslinked to the membrane immediately after transfer. Membranes were then hybridized in PerfectHyb Buffer (*Sigma*) at 68ºC for riboprobes and 43ºC in the case of oligoprobes. After hybridization, membranes were washed as previously described (Viegas *et al.*, 2007). Signals were visualized by PhosphorImaging (Storm Gel and Blot Imaging System,

Amersham Bioscience) and analyzed using the ImageQuant software (*Molecular Dynamics*).

RT-PCR reactions were performed using total RNA with the OneStep RT-PCR kit (*Quiagen*). Reactions were mainly carried out according to the supplier's instructions. Modifications were introduced regarding the amount of RNA and number of PCR cycles, depending on gene expression levels. The primer pair pIS-016/pIS017 was used to analyse *tig* expression. As a control, 16S rRNA was amplified with specific primers pIS-018/pIS-019. Prior to RT-PCR, all RNA samples were treated with Turbo DNA free Kit (*Ambion*). Control experiments, run in the absence of reverse transcriptase, yielded no product.

Hybridization probes

Primers for templates amplification are listed in Table S1 in the "Supplementary Information" section. Labelling of the riboprobes and oligoprobes were performed as described (Viegas *et al.*, 2007). The riboprobes were obtained using the primer pair pIS-021/pIS-022 for SraL riboprobe and pIS-017/pIS-020 for *tig* riboprobe. 5S rRNA and 16S rRNA were detected by the 5'-end-labelled oligonucleotides pIS-023 and pIS-024, respectively.

Protein extraction and Western Blot analysis

Bacteria were resuspended in the appropriate volume of Laemmli sample buffer (1.3% SDS, 10%, v/v, glycerol, 50 mM Tris/HCl, 1.8% β-mercaptoethanol, 0.02% bromophenol blue, pH 6.8) to get ≈ 10^7 bacteria per µl. RpoS protein was detected using the mouse monoclonal anti-sigma S 1RS1 antibody (*Santa Cruz Biotechnology*) at 1:5,000 dilution in antibody dilution buffer (50 mM Tris-HCl pH 7.5, 0.1% Tween-20, 3% BSA, 1mM sodium azide), and a goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (*Bio-Rad Life*). For recognition of the chaperonin GroEL, an anti-GroEL rabbit polyclonal antibody

was used (dilution 1:10,000, *Sigma*) and a goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (*Bio-Rad Life*). Membranes were developed with 1/10 diluted ECL prime reagent (*GE healthcare*) and visualized using the ChemiDoc XRS+ imaging system and the Quantity One software (*Bio-Rad Life*).

β-galactosidase assays

β-galactosidase activity was determined essentially as firstly described by Miller with minor modifications (Maloy, 1990). In brief, 100 μl of culture was added to 655 μl of cold buffer Z (100 mM Na_2HPO_4/NaH_2PO_4 pH 7, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol), and chloroform-SDS was used to permeabilize the cells. The reaction was started by the addition of the chromogenic substrate ortho-Nitrophenyl-β-galactoside (ONPG) to a final concentration of 0.8 mg/ml, conducted until it reaches a pale yellow colour at 30ºC and stopped with Na_2CO_3. Prior to recording absorbance at 420 nm samples were cleared by centrifugation. Optical density of the bacterial culture was also recorded at the time of the extraction of the sample. β-galactosidase activity in Miller units was calculated as follows: $(1{,}000 * A_{420}) / (t * v * OD_{600})$, where t corresponds to the reaction time in minutes and v to the sample volume in ml.

Chromatin immunoprecipitation (ChIP) assays

10 ml of overnight grown wild–type SL1344 and isogenic *rpoS* mutant cultures were exposed to 150 μg/ml rifampicin for 30 minutes to trap RNA polymerase at gene promoters. Cells were subjected to chemical crosslinking *in vivo* by adding formaldehyde and phosphate buffer pH 7.6 to a final concentration of 1% and 10 mM, respectively. Non-crosslinked control samples of both strains were processed in parallel. Crosslinking was left to proceed for 30 minutes at 37ºC with shacking and then quenched with 100 mM glycine for 30

min at 4ºC. Bacteria were recovered by centrifugation and washed twice with cold PBS. Bacterial pellets were resuspended in 1 mg/ml lysozyme in 0.2X IP buffer containing EDTA-free protease inhibitors cocktail (*Roche*) and maintained for 10 minutes at 37ºC. One volume of 2X IP buffer (200 mM Tris-HCl pH 8.0, 600 mM NaCl, 4% Triton X-100) was added, and the samples sonicated in a B. Braun sonifier (Labsonic U model; duty cycle 0.7, output 0.49). DNA in cleared lysates was further digested with 0.1 U of micrococcal nuclease (*New England Biolabs*) and 0.5 μg of RNase A in the presence of 5 mM $CaCl_2$ and 0.1 mg/ml BSA for 10 minutes at 37ºC. The digestion was stopped with 10 mM EDTA. DNA shearing was followed by agarose electrophoresis after reverse the crosslinking of an aliquot for 6 h at 65ºC.

Prior to immunoprecipitation 1/10 volume of the total extract was taken to be used as input sample control. The extracts were then pre-cleared with 20 μl of a 50% slurry containing 1:1 mix of protein-A and protein-G sepharose (*Sigma*) in 1X IP buffer for 4 hours at 4ºC with rotation. Immunoprecipitation was carried out with 2 μl of monoclonal mouse anti-sigma S 1RS1 antibody (*Santa Cruz Biotechnology*) overnight at 4ºC. All samples (no-antibody or pre-clearing controls and immunoprecipitates) were washed once with LiCl wash buffer (250 mM LiCl, 100 mM Tris-HCl pH 8, 2% TritonX-100), twice with 0.6 M NaCl buffer (100 mM Tris-HCl pH 8, 600 mM NaCl, 2% TritonX-100), twice with 1X IP buffer and once with TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). To elute complexes from the protein-A and G sepharose, beads were resuspended in 30 μl of ChIP elution buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS) and incubated for 30 min at 65ºC. The complexes were then incubated 6h at 65ºC to reverse crosslinking. Half of the sample was used to assess the efficiency of the immunoprecipitation by western blot. DNA was obtained from the other half of the sample by phenol/chloroform extraction, precipitated with isopropanol using 20 μg of

glycogen (Roche) as a carrier and the pellet dissolved in 12 μl of nuclease-free water.

For real-time quantitative PCR analysis of target DNA enrichment a 1/50 dilution sample of IP and no-antibody control were used as template. In the case of input and flowthrough samples we used a 1/200 dilution. Reactions were performed with the Power Sybr Green PCR master mix (*Applied Biosystems*) in a 10 μl final volume, and run in an ABI Prism 7,500 instrument (*Applied Biosystems*) using standard reaction conditions recommended by the manufacturer (10 min at 95°C; 45 cycles of 15 sec at 95°C and 1 min at 60°C; dissociation curve of 15 sec at 95°C, 1 min at 60°C and a progressive temperature increase until 95°C). Each sample was run in triplicate. Oligonucleotides osmY-F, osmY-R, sraL-F, sraL-R, rnpB-F, rnpB-R, 16S-F and 16S-R were used to amplify the corresponding target DNA at 0.5 μM final concentration and are included in the Supplementary Table S1 in the "Supplementary Information" section. For data analysis, the mean Ct value of technical replicates showing a standard deviation below 0.1 for target DNA was normalized to the mean Ct for *rrs* (16S) in the same sample (Ct_{target}-Ct_{rrs}). These values were referred to wild-type input sample and the anti-logarithm calculated.

Sequence retrieval and alignments

BlastN was used for sequence retrieval (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) of the following genome sequences: *Salmonella enterica* serovar Typhimurium LT2 (NC_003197), *Salmonella enterica* serovar Typhi Ty2 (NC_004631), *Salmonella bongori* NCTC 12419 (NC_015761), *Shigella boydii* CDC 3083-94 (NC_010658), *Shigella flexneri* 2a str. 301 (NC_004337), *Shigella dysenteriae* Sd197 (NC_007606), *Escherichia coli K12* (NC_000913), *Citrobacter rodentium* ICC168 (NC_013716), *Citrobacter koseri* ATCC

BAA-895 (NC_009792), *Enterobacter sp.* 638 (NC_009436), *Klebsiella pneumoniae* 342 (NC_011283). Alignments were made using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

RESULTS

SraL sRNA is directly regulated by RpoS (σ^S)

Since SraL sRNA is conserved among Enterobacteriaceae and its expression is induced preferentially in stationary phase (see Chapter 3) we hypothesized that it could be part of the general stress response orchestrated by the sigma factor RpoS (σ^S) of the RNA polymerase that operates in this growth phase. To this aim, we first examined the *sraL* promoter in search of conserved sequence elements that show specific features of promoters of *bona fide* RpoS-regulated genes. From the alignment of the immediately 75 nt upstream sequence of *sraL* in several enteric bacteria, we noticed some traits that are characteristic of an RpoS-regulated promoter (Figure 2A) (Typas *et al.*, 2007). In this regard, we observed a conserved -10 box that fits well with the consensus sequence retrieved from experimentally determined RpoS-regulated genes including the A/T-rich motif downstream the -10 box (Figure 2A) (Typas *et al.*, 2007; Weber *et al.*, 2005). Moreover, the -35 box is also characteristic of an RpoS-regulated promoter. These observations suggested a plausible selectivity of RpoS for the *sraL* promoter.

To test experimentally the putative RpoS-dependence on SraL expression, we compared SraL levels between the wild-type, an isogenic *rpoS* null mutant and a complemented *rpoS* mutant strain throughout stationary phase, the growth condition where SraL is highly expressed. Results presented in Figure 2B (upper panel) show that SraL sRNA is almost completely absent in the *rpoS* null mutant in this growth condition. In fact, reverse transcription and real-time quantitative

PCR revealed that in stationary phase SraL is about 500 times less abundant in the *rpoS* mutant than in the wild-type strain (data not shown). Consistently, SraL expression is partially restored in the *rpoS* mutant upon ectopic expression of a wild-type *rpoS* allele from a constitutive promoter (Figure 2B, upper panel). This might be due to the fact that ectopic RpoS is expressed at a much lower level in the complemented strain than the endogenous one in the wild-type (Figure 2B, lower panel). Even so, these low levels of RpoS protein suffice to restore SraL expression (Figure 2B). These results indicate that SraL expression in stationary phase is strictly dependent on RpoS activity.

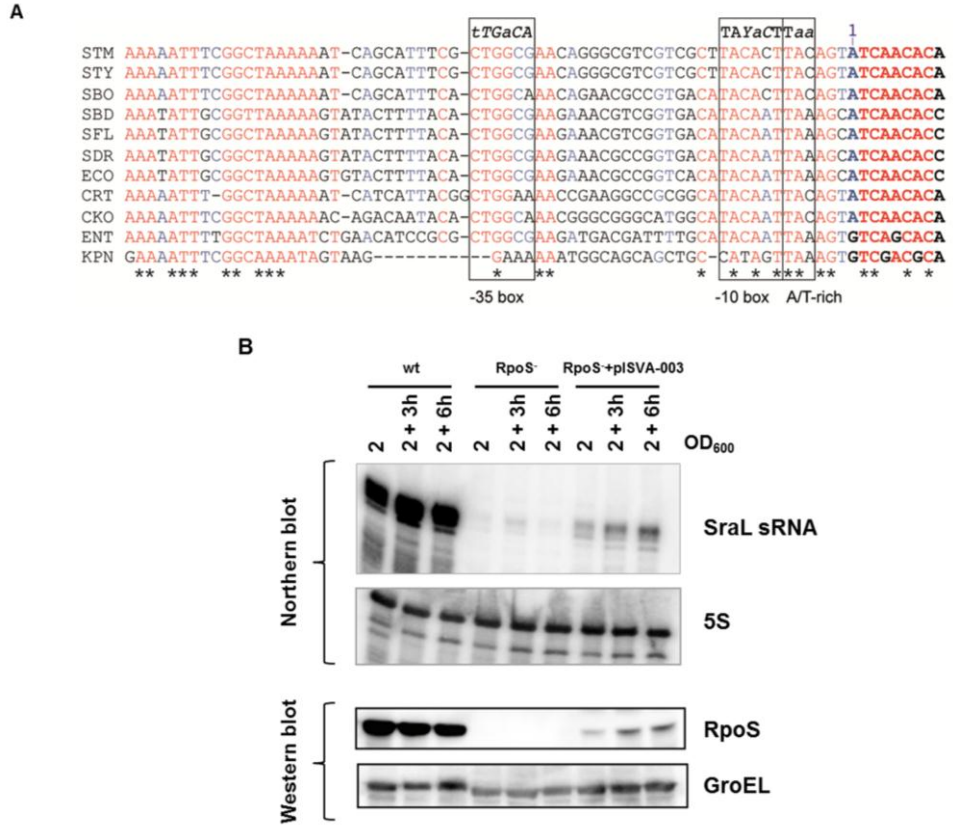

FIGURE 2 - Analysis of SraL sRNA regulation by RpoS. (A) Detail of the alignment of *sraL* gene of different Enterobacteriaceae (5'-UTR region). Consensus sequences of -10 and -35 boxes of RpoS, and RpoS-specific promoter sequence features located around the -10

core promoter element (A/T-rich discriminator) are indicated (Typas *et al.*, 2007). Parts of the -35 and -10 elements that are often degenerate in RpoS-dependent promoters are shown in italics (the least conserved nucleotides in lower case letters) (Typas *et al.*, 2007). Y: represents a C or a T. STM: *Salmonella enterica* serovar Typhimurium; STY: *Salmonella enterica* serovar Typhi; SBO: *Salmonella bongori*; SBD: *Shigella boydii* CDC 3083-94; SFL: *Shigella flexneri* 2a str. 301; SDR: *Shigella dysenteriae* Sd197; ECO: *Escherichia coli* K12; CRT: *Citrobacter rodentium* ICC168; CKO: *Citrobacter koseri*; ENT: *Enterobacter sp.* 638; KPN: *Klebsiella pneumoniae* 342. **(B)** SraL expression is dependent on RpoS. **(Upper panel)** SraL levels were detected by Northern blot using 15 μg of total RNA isolated at indicated time points during growth from *S.* Typhimurium wild-type (wt) and *rpoS* mutant strains and *rpoS* mutant strain carrying a constitutive plasmid expressing the wild-type *rpoS* allele (pISVA-003); 5S rRNA was used as loading control. **(Lower panel)** RpoS protein expression was monitored by Western blot analysis. Samples correspond to 5 x 10^7 bacteria at the indicated time points. GroEL was used as a loading control.

To further examine the SraL regulation by RpoS, we analyzed *sraL* promoter response in a transcriptional fusion to *lacZ* reporter gene in both wild-type and *rpoS* mutant genomic backgrounds. RpoS is known to be induced during entry into stationary phase and/or many other stress conditions. Thus, we first analyzed the transcriptional activity of *sraL* promoter in stationary phase, and observed a significantly lower *sraL* promoter-driven β-galactosidase activity when RpoS is not available (Figure 3A). To rule out any possible bias derived from the growth phase in which these analysis were performed, we investigated the RpoS-dependence of *sraL* expression under a stress condition which triggers RpoS-mediated response as it is high osmolarity (Hengge-Aronis *et al.*, 1993). Bacteria were grown to early exponential phase and then 0.5 M NaCl was added, maintaining the bacteria in these stress conditions for one hour. As a result of the increase in osmolarity, *sraL* transcriptional activity underwent an almost 3-fold induction in the wild-type strain while in the *rpoS* mutant strain the *sraL* promoter expression remained unchanged (Figure 3B). Consistently with our previous observations on the SraL expression pattern during bacterial growth, the transcriptional activity of *sraL* promoter is much higher in stationary phase (Figure 3A) than in exponential growth phase (Figure 3B). These data suggest that

the increase in SraL expression in stationary phase is the result of transcriptional regulation mediated by RpoS.

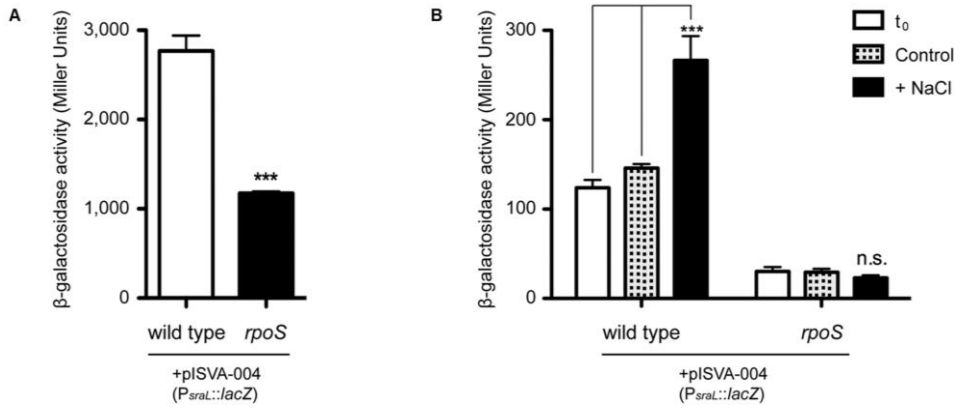

FIGURE 3 - Transcriptional response of *sraL* promoter to RpoS. (A) Transcriptional activity of *sraL* promoter in late stationary phase. Samples from overnight grown cultures of wild-type SL1344 and the isogenic *rpoS* null mutant transformed with a plasmid expressing the transcriptional fusion of *sraL* promoter to *lacZ* reporter gene (pISVA-004) were used to assess β-galactosidase activity. **(B)** Transcriptional activity of *sraL* promoter upon osmotic shock. Bacterial cultures were grown to reach exponential growth phase (OD 0.3), then NaCl was added to a final concentration of 0.5 M and let grow for one more hour. β-galactosidase activity was measured before (t_0, white bar) and after the treatment (+ NaCl, black bar). A control culture with no addition of NaCl was also carried in parallel (Control, light grey bar). Bars correspond to the mean ± standard deviation of three biological replicates. ***, $p<0.001$ by Student's t test; n.s., non-significant.

Up to now, our analysis supports that SraL expression is regulated by RpoS, but it does not differentiate between a direct or indirect regulation. To address this question, we analyzed *in vivo* the existence of binding of RpoS to the *sraL* promoter by chromatin immunoprecipitation assays (ChIP) (Raffaelle *et al.*, 2005). In this approach, complexes are chemically crosslinked *in vivo*, and the crosslinked RpoS-DNA complexes are further enriched by immunoprecipitation. The extent of *sraL* promoter enrichment in the immunoprecipitates (IPs), which is indicative of the binding *in vivo* of the sigma factor to the promoter, is determined by real-time quantitative PCR. We first confirmed the suitability and the

specificity of the monoclonal antibody for the immunoprecipitation of RpoS. Immunoprecipitation of un-crosslinked wild-type and *rpoS* mutant bacterial samples revealed that the antibody has a high affinity and specificity for RpoS, since no immunoreactive bands were visualized in *rpoS* mutant IPs, while a strong signal around the expected molecular weight for RpoS was obtained with the wild-type strain (Figure 4A). Two additional bands with a lower mobility were also immunoprecipitated (see asterisks in Figure 4A). Nevertheless, as they are not detected in the *rpoS* mutant input or IP samples, we reasoned that these immunoreactive bands might correspond to RpoS aggregates rather than an unspecific contaminating protein. These results confirm that the antibody displays a high affinity for RpoS and that it can be used to precipitate specifically DNA-RpoS complexes *in vivo* in ChIP assays. To assess the specificity of the ChIP assay, we first used *osmY* promoter as a target DNA sequence (Figure 4B). OsmY is a periplasmic protein of unknown function previously shown to be regulated by RpoS and we have used here as a positive control (Hengge-Aronis *et al.*, 1993; Yim *et al.*, 1994). Consistently, we found a 10-fold enrichment of *osmY* sequence in RpoS IPs, which indicate a relative high occupancy of *osmY* promoter by RpoS (Figure 4B). Interestingly, *sraL* target sequence was more than a hundred times enriched in RpoS IPs as compared to the input, which strongly supports the binding of RpoS to *sraL* promoter *in vivo* (Figure 4B). The higher enrichment of *sraL* promoter in RpoS IPs compared to that of *osmY* suggests that the transcriptional activity of *sraL* promoter is larger at the stationary phase, which points out the relevance of the induction of this sRNA at this specific growth phase. No enrichment in *rnpB* sequence, used here as a negative control, was observed in RpoS IPs. Collectively, these results strongly support that the increased expression levels of SraL sRNA observed during stationary phase result from a transcriptional induction directly mediated by the master regulator of the general stress response RpoS.

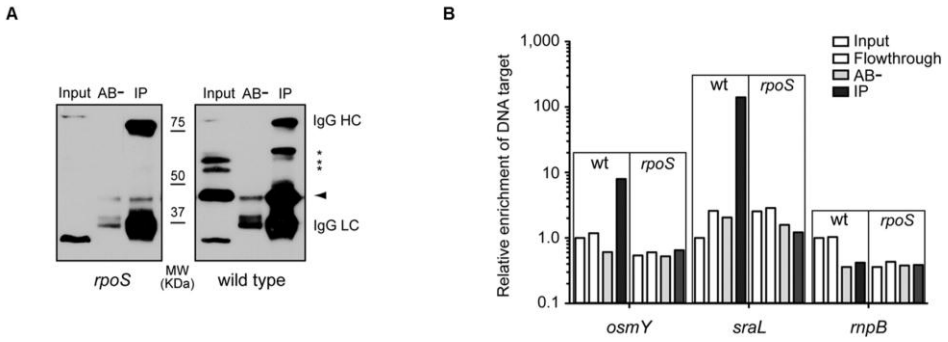

FIGURE 4 - ***In vivo*** **binding of RpoS to the** ***sraL*** **promoter at stationary phase. (A)** Anti-RpoS antibody immunoprecipitates the protein with high affinity and specificity. Western blot analysis using mouse monoclonal anti-RpoS antibody of protein samples coming from total extracts (Input) and immunoprecipitates either with the anti-RpoS antibody (IP) or with no antibody (AB^-). Protein extracts were obtained from wild-type SL1344 and the isogenic *rpoS* mutant strain. The arrowhead indicates the specific band corresponding to RpoS. Asterisks indicate other immunoreactive bands. IgG HC and LC indicate the heavy and light chains of the immunoglobulin used in the immunoprecipitation, respectively. **(B)** RpoS binds to *sraL* promoter *in vivo*. Chromatin immunoprecipitation (ChIP) assay with anti-RpoS antibody using the wild-type SL1344 (wt) and the isogenic *rpoS* mutant (*rpoS*) strains. DNA extracted from the samples was employed as a template for real-time quantitative PCR determination of target sequences (*osmY, sraL, rnpB*). The amount of target DNA was normalized to 16S (*rrs* genes) within each sample, and the relative enrichment is referred to the input sample of wild-type strain. Note that the relative enrichment is represented in log10 scale.

SraL sRNA down-regulates the expression of Trigger Factor mRNA

Although there are a few studies about SraL sRNA, the biological function of this sRNA was not yet revealed. The proteome of *S.* Typhimurium wild-type, *sraL* null mutant and *sraL* overexpressing strain was analysed in order to identify SraL putative targets. This analysis was performed using cells in late stationary phase of growth, the condition in which this sRNA is more expressed (see Figure 3 in Chapter 3) (Viegas *et al.*, 2007).

As presented in Chapter 3, a total of 713 proteins were identified and quantified across the three strains analyzed (see Supplementary Table S2 in "Supplementary Information" section in Chapter 3). Among these, Trigger Factor

(TF, *tig* (gene number STM0447)) was one of the proteins most affected by the change of SraL levels in the cell. Besides these proteomic observations, *tig* mRNA was predicted to base pair with SraL sRNA by the IntaRNA algorithm (http://www.bioinf.uni-freiburg.de/Software/) (Busch *et al.*, 2008). Trigger factor is found in all eubacteria and is the first chaperone encountered co-translationally by most of the nascent chains since it is localized in the exit of the ribosome tunnel (Stoller *et al.*, 1995). This localization enables its binding to nascent polypeptides and prevents improper intra- and/or inter-molecular interactions of the chains emerging on the surface of the ribosome (Valent *et al.*, 1995). TF was also shown to be a peptidyl-prolyl *cis*/*trans* isomerase (PPIase) and therefore accelerates proline-limited steps in protein folding with a very high efficiency (Hesterkamp *et al.*, 1996; Stoller *et al.*, 1995).

Since TF protein level is affected by SraL sRNA in the cell (about 2-fold difference between the deletion mutant and the overexpressing strain, see Table 3 in Chapter 3) we examined the *tig* mRNA level using the same strains. For that, total RNA was isolated from the wild-type cells and also from the mutant and overexpressing SraL strains in late stationary phase (OD2+6h). In agreement to the proteomic results, we could confirm by Northern blot and RT-PCR analysis that *tig* mRNA levels were in fact affected by the presence or absence of SraL sRNA (Figure 5, upper and middle panel). There was a 50% reduction of the *tig* mRNA levels when SraL is transcribed from an overexpressing plasmid. Moreover, when SraL is absent in the cell *tig* mRNA levels increase about 2-fold compared to the wild-type. Hereupon, SraL seems to negatively control either directly or indirectly the *tig* mRNA levels in the conditions tested.

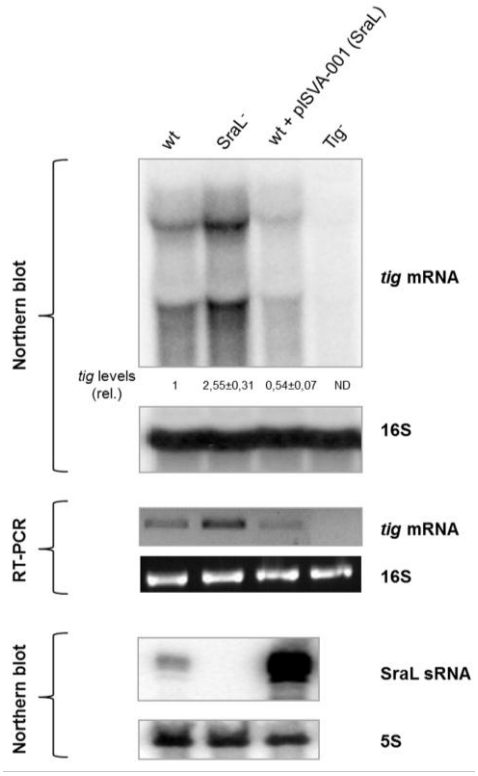

FIGURE 5 - Regulation of *tig* mRNA by SraL sRNA. Total cellular RNA was extracted from the *S.* Typhimurium strains indicated grown in LB at 37ºC till 6 h after OD_{600} of 2. **(Upper panel)** 15 μg of total RNA were separated on a 1.3% formaldehyde / agarose gel. The gel was then blotted to a Hybond-N^+ membrane and hybridized with the corresponding *tig* riboprobe. Full-length transcripts were quantified using a Molecular Dynamics PhosphorImager. The amount of RNA in the wild-type was set as one. The ratio between the amounts of RNA of each strain and the wild-type is represented (relative levels). A representative membrane is shown and values indicated correspond to the average of several Northern blot experiments with RNAs from at least two independent extractions. The membrane was stripped and then probed for 16S rRNA as loading control. (ND) Non-detectable. **(Middle panel)** RT-PCR experiments were carried out with specific primers for *tig* using 75 ng of total RNA extracted from the wild-type and derivatives, as indicated in each lane. RT-PCR primers specific for 16S rRNA shows that there were not significant variations in the amount of RNA used in each sample. **(Lower panel)** 15 μg of RNA were separated on a 6% PAA / 8.3 M urea gel. The gel was then blotted to a Hybond-N^+ membrane and hybridized with the corresponding SraL riboprobe. Probing for 5S rRNA confirmed equal loading.

SraL base pairs with Trigger Factor mRNA

To further investigate the role of SraL sRNA in the regulation of *tig* mRNA we performed a bioinformatic prediction to identify the interaction region between the sRNA and its target by using IntaRNA (Busch *et al.*, 2008) and RNAHybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) (Rehmsmeier *et al.*, 2004). Both algorithms were able to predict an imperfect SraL-*tig* interaction composed by two short segments (7 and 3 bp) (Figure 6A). Additionally, the predicted interaction between the sRNA and its target corresponds to a well conserved region in both RNAs (data not shown). To test whether pairing was direct and whether the predicted region was required, three base changes were introduced into a mutated version of SraL (SraL*) in the predicted base pairing region with the *tig* mRNA (Figure 6A). We ensured by bioinformatic predictions that these mutations do not modify the structure of the sRNA (compare the SraL and SraL* structures in Figure 6B). This mutation in the interaction site of the sRNA should obliterate the regulation of SraL over *tig* mRNA. In fact, the point mutations in the sRNA abolish the repression of *tig* mRNA (Figure 6C, upper panel). Furthermore, the Northern blot analysis presented in the Figure 6C (lower panel) shows that the point mutations do not compromise the expression of SraL sRNA. The results obtained provide additional evidence for the conclusion that SraL negatively regulates TF directly, by interacting with its mRNA.

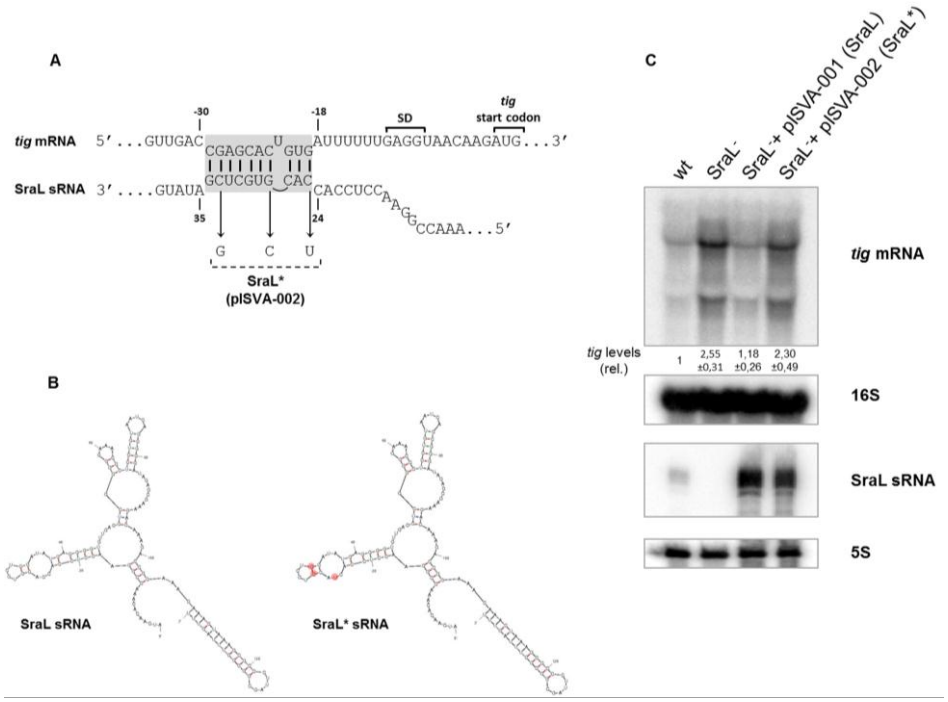

FIGURE 6 - Analysis of the interaction between SraL sRNA and *tig* mRNA. (A) Predicted interaction region between SraL sRNA and *tig* mRNA. The Shine-Dalgarno region and the start codon of *tig* are indicated. Point mutations to generate SraL* allele are indicated. **(B)** *S.* Typhimurium SraL and SraL* sRNA structures predicted by *Mfold* program (Zuker, 2003). The mutations inserted in SraL* are highlighted. **(C)** Mutations of SraL in the interaction region with the target validate SraL-*tig* interaction. *S.* Typhimurium *sraL* mutant cells carrying plasmids for the constitutive overexpression of either SraL or SraL* were grown until 6 h after OD_{600} of 2. **(Upper panel)** The expression level of *tig* mRNA was determined by using a 1.3% formaldehyde / agarose gel. The amount of RNA in wild-type was set as one. The ratio between the RNA amount of each strain and the wild-type is represented (relative levels). A representative membrane is shown and values indicated correspond to the average of several Northern blot experiments with RNAs from at least two independent extractions. The membrane was stripped and then probed for 16S rRNA as loading control. **(Lower panel)** 15 μg of RNA were separated on a 6% PAA / 8.3 M urea to determine the expression level of both SraL and SraL*; probing of 5S rRNA was used as a loading control.

DISCUSSION

Trans-encoded sRNAs are known to regulate several genes involved in stress responses. Computational and experimental methodologies have allowed the association of several of these sRNAs with important regulons of both *E. coli* and *Salmonella*. The RpoS regulon includes genes with functions in carbon metabolism, stress resistance, cell envelope integrity, morphology, stationary phase, and virulence (Battesti *et al.*, 2011; Dong and Schellhorn, 2010). In this report we have included the SraL sRNA in the RpoS regulon since SraL was shown to be directly regulated by this sigma factor. An RpoS-recognized promoter is normally identified by a series of characteristic features (Typas *et al.*, 2007). The predicted SraL promoter region (Argaman *et al.*, 2001) presents several of these features namely the -35 and -10 box and the extended -10 motif TAA. Moreover, these features are also present in the several enteric bacteria analyzed. Accordingly, it is possible to admit that besides its expression in several other enterobacterial species this sRNA is also directly transcribed by RpoS in these bacteria. There are only a few studies reporting the control of other sRNAs by RpoS (Opdyke *et al.*, 2004; Padalon-Brauch *et al.*, 2008). However, up to now there is only the case of SdsR sRNA (that controls the synthesis of the major *Salmonella* porin OmpD) which is controlled by RpoS and is conserved in a broad range of enteric bacteria (Fröhlich *et al.*, 2012). Therefore, SraL constitutes the second example of a conserved sRNA that is controlled by RpoS. In previous work we had shown that SraL is post-transcriptionally controlled by ribonucleases (PNPase and the degradosome complex) and also by polyadenylation (Viegas *et al.*, 2007). Therefore, after this report we can conclude that SraL is a tightly regulated sRNA both at transcriptional and post-transcriptional levels.

After the discovery of MicF sRNA and subsequent unravelling of its function (Mizuno *et al.*, 1984) more than one hundred sRNAs were identified.

However, the biological function of many of these sRNAs is still unknown. In this study we present for the first time a target for SraL sRNA. We show that SraL contributes to the regulation of the expression of the chaperone trigger factor in late stationary phase. SraL inhibits *tig* expression at the post-transcriptional level by an antisense mechanism that implicates the base pairing between a region in the 5'-end of SraL and a few nucleotides before the ribosome binding site of the *tig* mRNA. This interaction region between the sRNA and its target was confirmed by mutations in SraL sRNA that abolished the regulation. Unlike what it happens in many cases of riboregulation, the region of interaction between SraL sRNA and *tig* mRNA does not overlap the ribosome binding site and the *tig* mRNA start codon. However, interactions involving nucleotides in the mRNA leader in the vicinity of the ribosome binding site and/or the start codon have been also shown to inhibit translation (Babitzke and Gollnick, 2001; Chen *et al.*, 2004; Liu *et al.*, 1997). Therefore, it is plausible to assume that it is also the case in this regulation.

Trigger factor is one of the three major chaperones (along with DnaK and GroEL) which cooperate in the folding of the newly synthesized cytosolic proteins (Deuerling *et al.*, 1999; Kandror *et al.*, 1995; Lecker *et al.*, 1989; Stoller *et al.*, 1995). Moreover, it was very recently reported that this chaperone can also unfold preformed structures and reverse premature misfolds, giving nascent chains a new opportunity for productive folding (Hoffmann *et al.*, 2012). It possesses a peptidyl-prolyl *cis*/*trans* isomerase (PPIase) activity and accelerates proline-limited steps in protein folding with a very high efficiency (Stoller *et al.*, 1995). This reaction is often a rate-limiting step in the folding of certain polypeptides. Even though it is dispensable for growth, TF is a very important protein since it is the first chaperone encountered by the majority of nascent peptide chains due to its location in contact with the large subunit of the ribosomes (Stoller *et al.*, 1995).

Therefore, this protein associates co-translationally with most of the nascent polypeptides. TF competes with DnaK in the chaperoning of newly synthesized peptides (Deuerling *et al.*, 1999; Teter *et al.*, 1999) what justifies why it is not an essential protein. The importance of TF for metabolism of bacteria is indicated by the discovery of a *tig* gene in *Mycoplasma genitalium* (Bang *et al.*, 2000). This bacterium is believed to be free from genetic redundancy and thus contains only the minimal set of genes required for life. This chaperone appears to be the only PPIase of this organism (Bang *et al.*, 2000).

This study presents for the first time a regulatory role of SraL sRNA in *S.* Typhimurium. Despite some significant differences over the sequences of both SraL and *tig* genes in Enterobacteriaceae, the interaction region between the two RNAs corresponds to a very well conserved region. Thus, it is possible that this regulation of SraL sRNA over *tig* mRNA also occurs in many other enteric bacteria. The biological significance of the regulatory pathway involving SraL and TF is not totally clear. During stationary phase the overall rate of protein synthesis is reduced when compared to an exponentially growing culture (Albertson *et al.*, 1990; Kuzj *et al.*, 1998), concomitant with a decrease in ribosomes' levels (Lambert *et al.*, 1983). This happens because the cell avoids the production of unnecessary proteins when cells are not growing. Since TF is associated with the ribosomes and plays a key role in the folding of nascent peptides it is possible that it is less required in stationary phase. In fact, results from our lab have shown that *tig* mRNA levels are higher at exponential phase (data not shown). Since trigger factor is constitutively expressed in the cell, the RpoS induction of SraL sRNA under stationary phase seems to occur to avoid the superfluous production of this chaperone.

Since both SraL and TF are very well conserved in enterobacteria this report will have a significant impact in the field. Moreover, this study constitutes the first report connecting small RNAs with protein folding.

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REFERENCES

Albertson, N.H., Nyström, T., and Kjelleberg, S. 1990. Macromolecular synthesis during recovery of the marine *Vibrio* sp. S14 from starvation. *J Gen Microbiol*:2201-2207.

Argaman, L., Hershberg, R., Vogel, J., Bejerano, G., Wagner, E.G., Margalit, H., and Altuvia, S. 2001. Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli*. *Curr Biol*. 11:941-950.

Babitzke, P., and Gollnick, P. 2001. Posttranscription initiation control of tryptophan metabolism in *Bacillus subtilis* by the trp RNA-binding attenuation protein (TRAP), anti-TRAP, and RNA structure. *J Bacteriol*. 183:5795-5802.

Bang, H., Pecht, A., Raddatz, G., Scior, T., Solbach, W., Brune, K., and Pahl, A. 2000. Prolyl isomerases in a minimal cell. Catalysis of protein folding by trigger factor from *Mycoplasma genitalium*. *Eur J Biochem*. 267:3270-3280.

Battesti, A., Majdalani, N., and Gottesman, S. 2011. The RpoS-mediated general stress response in *Escherichia coli*. *Annu Rev Microbiol*. 65:189-213.

Busch, A., Richter, A.S., and Backofen, R. 2008. IntaRNA: efficient prediction of bacterial sRNA targets incorporating target site accessibility and seed regions. *Bioinformatics*. 24:2849-2856.

Chen, S., Zhang, A., Blyn, L.B., and Storz, G. 2004. MicC, a second small-RNA regulator of Omp protein expression in *Escherichia coli*. *J Bacteriol*. 186:6689-6697.

Datsenko, K.A., and Wanner, B.L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A*. 97:6640-6645.

Deuerling, E., Schulze-Specking, A., Tomoyasu, T., Mogk, A., and Bukau, B. 1999. Trigger factor and DnaK cooperate in folding of newly synthesized proteins. *Nature*. 400:693-696.

Dong, T., and Schellhorn, H.E. 2010. Role of RpoS in virulence of pathogens. *Infect Immun*. 78:887-897.

Fröhlich, K.S., Papenfort, K., Berger, A.A., and Vogel, J. 2012. A conserved RpoS-dependent small RNA controls the synthesis of major porin OmpD. *Nucleic Acids Res*. 40:3623-3640.

Gottesman, S., and Storz, G. 2011. Bacterial small RNA regulators: versatile roles and rapidly evolving variations. *Cold Spring Harb Perspect Biol*. 3.

Hengge-Aronis, R., Lange, R., Henneberg, N., and Fischer, D. 1993. Osmotic regulation of rpoS-dependent genes in *Escherichia coli*. *J Bacteriol*. 175:259-265.

Hesterkamp, T., Hauser, S., Lutcke, H., and Bukau, B. 1996. *Escherichia coli* trigger factor is a prolyl isomerase that associates with nascent polypeptide chains. *Proc Natl Acad Sci U S A*. 93:4437-4441.

Hoffmann, A., Becker, A.H., Zachmann-Brand, B., Deuerling, E., Bukau, B., and Kramer, G. 2012. Concerted Action of the Ribosome and the Associated Chaperone Trigger Factor Confines Nascent Polypeptide Folding. *Mol Cell*.

Hoffmann, A., Bukau, B., and Kramer, G. 2010. Structure and function of the molecular chaperone Trigger Factor. *Biochim Biophys Acta*. 1803:650-661.

Hoiseth, S.K., and Stocker, B.A. 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature*. 291:238-239.

Johansen, J., Eriksen, M., Kallipolitis, B., and Valentin-Hansen, P. 2008. Down-regulation of outer membrane proteins by noncoding RNAs: unraveling the cAMP-CRP- and sigmaE-dependent CyaR-*ompX* regulatory case. *J Mol Biol*. 383:1-9.

Johansen, J., Rasmussen, A.A., Overgaard, M., and Valentin-Hansen, P. 2006. Conserved small non-coding RNAs that belong to the sigmaE regulon: role in down-regulation of outer membrane proteins. *J Mol Biol*. 364:1-8.

Kandror, O., Sherman, M., Rhode, M., and Goldberg, A.L. 1995. Trigger factor is involved in GroEL-dependent protein degradation in *Escherichia coli* and promotes binding of GroEL to unfolded proteins. *EMBO J*. 14:6021-6027.

Kröger, C., Dillon, S.C., Cameron, A.D., Papenfort, K., Sivasankaran, S.K., Hokamp, K., Chao, Y., Sittka, A., Hébrard, M., Händler, K., Colgan, A., Leekitcharoenphon, P., Langridge, G.C., Lohan, A.J., Loftus, B., Lucchini, S., Ussery, D.W., Dorman, C.J., Thomson, N.R., Vogel, J., and Hinton, J.C. 2012. The transcriptional landscape and small RNAs of *Salmonella enterica* serovar Typhimurium. *Proc Natl Acad Sci U S A*. 109:E1277-1286.

Kuzj, A.E., Medberry, P.S., and Schottel, J.L. 1998. Stationary phase, amino acid limitation and recovery from stationary phase modulate the stability and translation of chloramphenicol acetyltransferase mRNA and total mRNA in *Escherichia coli*. *Microbiology*. 144 (Pt 3):739-750.

Lambert, J.M., Boileau, G., Howe, J.G., and Traut, R.R. 1983. Levels of ribosomal protein S1 and elongation factor G in the growth cycle of *Escherichia coli*. *J Bacteriol*. 154:1323-1328.

Lecker, S., Lill, R., Ziegelhoffer, T., Georgopoulos, C., Bassford, P.J., Jr., Kumamoto, C.A., and Wickner, W. 1989. Three pure chaperone proteins of *Escherichia coli* - SecB, trigger factor and GroEL - form soluble complexes with precursor proteins *in vitro*. *EMBO J*. 8:2703-2709.

Liu, M.Y., Gui, G., Wei, B., Preston, J.F., 3rd, Oakford, L., Yuksel, U., Giedroc, D.P., and Romeo, T. 1997. The RNA molecule CsrB binds to the global regulatory protein CsrA and antagonizes its activity in *Escherichia coli*. *J Biol Chem*. 272:17502-17510.

Lober, S., Jackel, D., Kaiser, N., and Hensel, M. 2006. Regulation of *Salmonella* pathogenicity island 2 genes by independent environmental signals. *Int J Med Microbiol*. 296:435-447.

Lutz, R., and Bujard, H. 1997. Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. *Nucleic Acids Res*. 25:1203-1210.

Maloy, S.R. 1990. Experimental Techniques in Bacterial Genetics. Jones and Bartlett Publishers, Boston.

Mecsas, J., Rouviere, P.E., Erickson, J.W., Donohue, T.J., and Gross, C.A. 1993. The activity of sigma E, an *Escherichia coli* heat-inducible sigma-factor, is modulated by expression of outer membrane proteins. *Genes Dev*. 7:2618-2628.

Missiakas, D., Betton, J.M., and Raina, S. 1996. New components of protein folding in extracytoplasmic compartments of *Escherichia coli* SurA, FkpA and Skp/OmpH. *Mol Microbiol*. 21:871-884.

Mizuno, T., Chou, M.Y., and Inouye, M. 1984. A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). *Proc Natl Acad Sci U S A*. 81:1966-1970.

Opdyke, J.A., Kang, J.G., and Storz, G. 2004. GadY, a small-RNA regulator of acid response genes in *Escherichia coli*. *J Bacteriol*. 186:6698-6705.

Ortega, A.D., Gonzalo-Asensio, J., and García-Del Portillo, F. 2012. Dynamics of *Salmonella* small RNA expression in non-growing bacteria located inside eukaryotic cells. *RNA Biol*. 9.

Padalon-Brauch, G., Hershberg, R., Elgrably-Weiss, M., Baruch, K., Rosenshine, I., Margalit, H., and Altuvia, S. 2008. Small RNAs encoded within genetic islands of *Salmonella typhimurium* show host-induced expression and role in virulence. *Nucleic Acids Res*. 36:1913-1927.

Papenfort, K., Pfeiffer, V., Mika, F., Lucchini, S., Hinton, J.C., and Vogel, J. 2006. SigmaE-dependent small RNAs of *Salmonella* respond to membrane stress by accelerating global *omp* mRNA decay. *Mol Microbiol*. 62:1674-1688.

Podkovyrov, S.M., and Larson, T.J. 1995. A new vector-host system for construction of *lacZ* transcriptional fusions where only low-level gene expression is desirable. *Gene*. 156:151-152.

Raffaelle, M., Kanin, E.I., Vogt, J., Burgess, R.R., and Ansari, A.Z. 2005. Holoenzyme switching and stochastic release of sigma factors from RNA polymerase *in vivo*. *Mol Cell*. 20:357-366.

Raivio, T.L., and Silhavy, T.J. 1999. The sigmaE and Cpx regulatory pathways: overlapping but distinct envelope stress responses. *Curr Opin Microbiol*. 2:159-165.

Rehmsmeier, M., Steffen, P., Höchsmann, M., and Giegerich, R. 2004. Fast and effective prediction of microRNA/target duplexes. *RNA*. 10:1507-1517.

Schmieger, H. 1971. The fate of the bacterial chromosome in P22-infected cells of *Salmonella typhimurium*. *Mol Gen Genet*. 110:238-244.

Sittka, A., Pfeiffer, V., Tedin, K., and Vogel, J. 2007. The RNA chaperone Hfq is essential for the virulence of *Salmonella typhimurium*. *Mol Microbiol*. 63:193-217.

Stoller, G., Rücknagel, K.P., Nierhaus, K.H., Schmid, F.X., Fischer, G., and Rahfeld, J.U. 1995. A ribosome-associated peptidyl-prolyl cis/trans isomerase identified as the trigger factor. *EMBO J*. 14:4939-4948.

Storz, G., Vogel, J., and Wassarman, K.M. 2011. Regulation by small RNAs in bacteria: expanding frontiers. *Mol Cell*. 43:880-891.

Teter, S.A., Houry, W.A., Ang, D., Tradler, T., Rockabrand, D., Fischer, G., Blum, P., Georgopoulos, C., and Hartl, F.U. 1999. Polypeptide flux through bacterial Hsp70: DnaK cooperates with trigger factor in chaperoning nascent chains. *Cell*. 97:755-765.

Tierrez, A., and Garcia-del Portillo, F. 2004. The *Salmonella* membrane protein IgaA modulates the activity of the RcsC-YojN-RcsB and PhoP-PhoQ regulons. *J Bacteriol*. 186:7481-7489.

Typas, A., Becker, G., and Hengge, R. 2007. The molecular basis of selective promoter activation by the sigmaS subunit of RNA polymerase. *Mol Microbiol*. 63:1296-1306.

Udekwu, K.I., and Wagner, E.G. 2007. Sigma E controls biogenesis of the antisense RNA MicA. *Nucleic Acids Res*. 35:1279-1288.

Valent, Q.A., Kendall, D.A., High, S., Kusters, R., Oudega, B., and Luirink, J. 1995. Early events in preprotein recognition in *E. coli*: interaction of SRP and trigger factor with nascent polypeptides. *EMBO J*. 14:5494-5505.

Viegas, S.C., Pfeiffer, V., Sittka, A., Silva, I.J., Vogel, J., and Arraiano, C.M. 2007. Characterization of the role of ribonucleases in *Salmonella* small RNA decay. *Nucleic Acids Res*. 35:7651-7664.

Wang, R.F., and Kushner, S.R. 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene*. 100:195-199.

Wassarman, K.M., Repoila, F., Rosenow, C., Storz, G., and Gottesman, S. 2001. Identification of novel small RNAs using comparative genomics and microarrays. *Genes Dev*. 15:1637-1651.

Weber, H., Polen, T., Heuveling, J., Wendisch, V.F., and Hengge, R. 2005. Genome-wide analysis of the general stress response network in *Escherichia coli*: sigmaS-dependent genes, promoters, and sigma factor selectivity. *J Bacteriol*. 187:1591-1603.

Yim, H.H., Brems, R.L., and Villarejo, M. 1994. Molecular characterization of the promoter of *osmY*, an rpoS-dependent gene. *J Bacteriol*. 176:100-107.

Zuker, M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*. 31:3406-3415.

SUPPLEMENTARY INFORMATION

Supplementary Tables

SUPPLEMENTARY TABLE S1 - List of oligonucleotides used in this work. The restriction and T7 sequences in the primers used for cloning procedures and for riboprobe synthesis, respectively, are shown in bold and underlined.

Oligo	Sequence 5' to 3'
pIS-001	ATTTCGGCTAAAAAATCAGCATTTCGCTGGCGAACAGGGCGTCGTCGCTTAGTGTAGGCTGGAGCTGCTTC
pIS-002	CATGCACTCGGCCATCGGGCTGAGCTCACCTAAAACTAAAGCGCCGCTAAGTGGTCCATATGAATATCCTCCTTAG
pIS-003	GTTTTTCTCGAGATGCGCTGCGAAC
pIS-004	GTTTTTTCTAGAAGATGATTAACATGCACTCG
pIS-005	CGAAGCAAATAGCACGTGCTTGCGGAGTAGAGTTGACCGAGCACTGTGATGTGTAGGCTGGAGCTGCTTC
pIS-006	CGTCACTGAAAGGTGACGGGTTTTTGTGCAAATTTCGTGCTTTTAACGCGGGTCCATATGAATATCCTCCTTAG
pIS-007	GACGACAGGGAATGTGATTG
pIS-008	GCTCTAACGCTAACACTG
pIS-009	ATCAACACAAACCGGAACCTC
pIS-010	**GTTTTTGGTACC**CACTCGGCCATCGGGCTG
pIS-011	ATCAACACAAACCGGAACCTCCACTACCTGCTGGATATGAGGGGTGTTGACGTC
pIS-012	**GTTTTTTCTA**GAGCCACCTTTTGAGTCAGAATACGC
pIS-013	**GTTTTTTAAGC**TTGACAAGGGTACTTACTCGC
pIS-014	**GTTTTTCTA**GAACGCCAGCTCTTCCAGCGCCCACA
pIS-015	**GTTTTGGATCC**GTGTTGATACTGTAAGTGTAAGCG
pIS-016	CAGCTCCAGAGCCTGTTTC
pIS-017	GAAGAGTTCGAAGGCGGCAAAG
pIS-018	AGGCGGTCTGTCAAGTCGGATG
pIS-019	ACAGCCATGCAGCACCTGTCTC
pIS-020	**GTTTTTTTTTTAATACGACTCACTATAGG**GTCAGTTCCGGCAGTTCGCGTTCTTC
pIS-021	ATCAACACAAACCGGAAC
pIS-022	**GTTTTTTTTTTAATACGACTCACTATAGG**GAGGTAAGGGCGCTTTAGTTTG
pIS-023	CTACGGCGTTTCACTTCTGAGTTC
pIS-024	ACGGCTACCTTGTTACGACTT
osmY-F	CATTCAGCAATGCAACCTCG
osmY-R	TTTGCTCGTAATTTGAGCTCAGG
sraL-F	AAAATCAGCATTTCGCTGGC
sraL-R	ATACTGTAAGTGTAAGCGACGACGC
16S-F	CCTGGGAACTGCATTCGAA
16S-R	TGGAATTCTACCCCCCTCTACA
rnpB-F	CCCCTATTTGGCCTTGCT
rnpB-R	GTGAAAGGGTGCGGTAAGAG

Chapter 5

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

During the lifecycle of any organism, either prokaryotic or eukaryotic, the cells have to adjust protein synthesis in response to changes in the environment. The control of gene expression is crucial for adaptation, and it can occur at the transcriptional, post-transcriptional, translational and post-translational levels. This Dissertation is mainly focused on the post-transcriptional regulation of gene expression in *Salmonella* Typhimurium.

Ribonucleases are the key enzymes responsible for the maturation, degradation and quality control of the RNAs (Arraiano *et al.*, 2010). All organisms studied contain many RNases of many different classes, showing that these are very ancient and important processes. RNA degradation (one of the themes addressed in this Dissertation) is an essential function not only for the regulation of gene expression but also for the recycling of nucleotides.

Until recently, tRNAs and rRNAs were considered the only non-coding RNA species. However, in the last decades it was discovered the existence of other type of RNAs called small non-coding RNAs. The majority of these sRNAs does not encode proteins and act as regulators of gene expression. However, there are cases of bifunctional sRNAs that act not only as riboregulators but also serve as mRNA templates for functional proteins (Vanderpool *et al.*, 2011). There are several types of sRNAs in prokaryotic organisms that are divided in different classes depending on their mode of action. This Dissertation comprises work related with two sRNAs very well conserved in enteric bacteria, MicA and SraL.

MicA is a *trans*-encoded sRNA transcribed by the sigma factor σ^E upon stress conditions that unbalance OMP levels (Figueroa-Bossi *et al.*, 2006; Johansen *et al.*, 2006; Papenfort *et al.*, 2006; Udekwu and Wagner, 2007). There were two targets described for this sRNA in *Salmonella*, the outer membrane protein *ompA* and the maltoporin *lamB*. In a previous work, we have constructed several ribonuclease mutants in *Salmonella* since no RNase mutants were available in this

organism. We have analyzed the role of several RNases in the degradation of different sRNAs (Viegas *et al.*, 2007). In order to follow our previous work, we have further evaluated the importance of the endoribonucleases E and III in the degradation of MicA sRNA. RNase E has been seen as the most important enzyme in the sRNA-mediated destabilization of target mRNAs (Fröhlich *et al.*, 2012; Kawamoto *et al.*, 2006; Massé *et al.*, 2003; Pfeiffer *et al.*, 2009). However, reports on the important role of RNase III in this process are increasing (Kaberdin and Blasi, 2006; Lasa *et al.*, 2012). By several *in vitro* and *in vivo* experiments, this ds-specific RNase was shown to be crucial for the degradation of MicA when it is coupled with its targets known in *Salmonella*. Moreover, even though MicA exhibits two stem-loop structures that could be substrates of RNase III, this endoribonuclease is not able to cleave free MicA. This mechanism constitutes the first example of a system controlled by an Hfq-dependent *trans*-encoded sRNA that involves the coupled degradation of the sRNA with its mRNA(s) target(s) by RNase III. Interestingly, Hfq was shown to be essential for the efficient RNase III cleavage of the duplex formed between MicA and its target(s) (Andrade *et al.*, 2012). This fact is explained by the reduced number of duplexes formed between the sRNA and its target(s) in the absence of Hfq. Our previous results had shown that an *rne* mutation impairing the degradosome formation strongly increased the stability of MicA (Viegas *et al.*, 2007). Our *in vitro* results obtained using the purified RNase E from *Salmonella*, confirmed that in fact RNase E is directly involved in the degradation of free MicA. In summary, the first part of the work of this Dissertation allowed the proposition of a model for MicA decay, in which two different endoribonucleases exhibit different roles: RNase III is involved in MicA degradation when it is coupled with its target(s); and RNase E participates in the target-independent pathway.

MicA has been extensively studied in the last years. Several targets were described for this sRNA in *E. coli* (Bossi and Figueroa-Bossi, 2007; Coornaert *et al.*,

2010; Gogol *et al.*, 2011; Udekwu, 2010; Udekwu *et al.*, 2005). Indeed, this is the only sRNA known to control targets both in *trans* and in *cis* (Udekwu, 2010). MicA down-regulates targets with different functions in the cell including several outer membrane proteins and lipoproteins associated with the cell envelope, the PhoPQ TCS that regulates genes involved in several pathways, and an enzyme responsible for the synthesis of AI-2 a signalling molecule used in quorum sensing. Curiously, this sRNA was also associated with the correct biofilm formation in *Salmonella* Typhimurium. It was shown that balanced MicA levels are essential for mature *Salmonella* biofilm formation (Kint *et al.*, 2010). The two MicA targets described in *Salmonella, ompA* and *lamB* mRNAs, were discovered in very specific conditions (Bossi and Figueroa-Bossi, 2007). Since MicA is significantly expressed in SPI-1 and SPI-2 inducing conditions it is probably involved in virulence. Actually, a recent report determined the role of MicA in *Salmonella* Typhimurium virulence. Surprisingly, the *micA* mutant strain was shown to be more virulent in mouse model than the wild-type strain (Homerova *et al.*, 2011). The authors of this manuscript proposed that a possible reason for this result is the fact that σ^E is activated in the *micA* mutant and this sigma factor is essential for *Salmonella* infection. More work is obviously needed for a complete understanding of MicA function in *Salmonella* virulence. In fact, the major target of this sRNA, OmpA, was already described to be a virulence factor of several bacterial organisms, namely *E. coli*, *Yersinia pestis* and also *S.* Typhimurium (Bartra *et al.*, 2012; Lee *et al.*, 2010; Wu *et al.*, 2009). Moreover, other described target of MicA, the mediator of the quorum sensing mechanism LuxS, was very recently shown to be important for the virulence of the avian pathogenic *E. coli* (Han *et al.*, 2012). Taking into consideration the results obtained, a possible project for the near future will be the identification of MicA targets using SPI-1 and SPI-2 inducing conditions in order understand the role of MicA in *Salmonella* virulence. *Salmonella* was shown to be able to invade non-phagocytic epithelial cells (Jepson

et al., 1995). Another important study will be to determine the effect of *micA* mutation on the invasion rate of this type of cells *in vitro*. *Salmonella* survival in the host is also dependent on the ability to survive and replicate inside macrophages. Thus, we also intend to study the role of MicA in macrophage survival. All these studies will help us to understand in more detail the role of MicA in *Salmonella* virulence.

The numerous genome-wide searches performed in the last decades enabled the identification of a plethora of new sRNAs in several growth conditions. However, the biological role of numerous of these sRNAs is not yet revealed. SraL is one of these sRNAs that was detected in two global genetic studies in *E. coli* and which function was unknown. A major challenge in sRNA research concerns the identification of sRNA targets. Experimental approaches for this detection include standard genetic screens, gene knockouts and overexpression of the sRNA of interest, followed by proteomic and/or transcriptomic analyses. To complement the experimental procedures, several methods for highly sensitive bio-computational target prediction were developed. These algorithms either search for complementarity between sRNA and mRNA sequences or try to minimize the free energy of the hybridization between the two RNAs (Busch *et al.*, 2008; Muckstein *et al.*, 2006; Tjaden, 2008; Zhang *et al.*, 2006). Most of the algorithms use additional data, such as the secondary structure of the sRNA and/or the target, or the nucleotide composition of the interacting regions. Even though these software programs consider several important parameters, they are not 100% accurate and originate many false positive and negative results. During the work developed in this Dissertation we have used several bioinformatic tools to try to predict SraL targets. However, we had to resort to a proteomic analysis to narrow the number of the putative targets. The conjugation of the data obtained using both bioinformatic and proteomic analyses strengthened our hypothesis.

Several SraL sRNA putative targets were identified in late stationary phase of growth. Some of the proteins most affected directly or indirectly by the different levels of SraL expression correspond to enzymes or subunits of enzymes that participate in crucial steps of carbohydrate metabolism. All organisms depend on carbohydrates, which provide cells with energy and the building blocks for biosynthesis of all macromolecules. Thus, it is predictable that all the pathways involved in metabolism of carbohydrates are tightly regulated. Moreover, organisms usually express the genes required for the utilization of a particular sugar substrate only if it is available in the environment. In fact, there are several sRNAs already implicated in the control of the sugar metabolism at different levels (Görke and Vogel, 2008).

One of the putative targets that evoke our attention, not only by the difference in both protein and mRNA expression but also by its important cellular role, was the chaperone Trigger Factor (TF). Newly synthesized polypeptide chains of cytosolic proteins have the potential to start the folding process co-translationally (Hartl and Hayer-Hartl, 2009). However, within the cellular environment they interact with a large number of molecular chaperones that guide the folding process. Two groups of chaperones assist the *de novo* folding: ribosome-associated chaperones that interact early with nascent chains and chaperones that do not associate with ribosomes and act later during translation or after polypeptide release (Kramer *et al.*, 2009). TF is the only ribosome-associated chaperone in bacteria and is found in all eubacteria analyzed. It accommodates the substrate in its interior, which provides a protective environment to prevent proteins from aggregating or degradation. In addition, TF can prevent premature and incorrect folding of proteins during synthesis (Preissler and Deuerling, 2012). During this Doctoral work, SraL was associated to this process. This sRNA represses *tig* expression at the post-transcriptional level possibly by competition with ribosome binding, as was shown to be the case for

several negatively acting sRNAs. This repression is probably accompanied by degradation of the mRNA. The physiological relevance of the degradation of target mRNAs should rely on making the gene silencing irreversible. In the near future, one of our aims will be to determine the RNases involved in the degradation of *tig* mRNA after the interaction with SraL sRNA. Moreover, the function of the Hfq protein in the *tig* regulation by SraL needs to be also addressed. In fact, this sRNA was previously shown to be destabilized in a strain lacking Hfq, indicating that this sRNA belongs to the group of Hfq-dependent sRNAs (Viegas *et al.*, 2007). Moreover, it was also previously reported that *tig* levels changed in an Hfq mutant strain in early stationary phase of growth (Sittka *et al.*, 2008).

The interaction region between SraL and *tig* mRNA was bioinformatically predicted and confirmed by the insertion of mutations in the sRNA. This area is often called seed region. The optimal length and nucleotide composition of the bacterial seed have not been fully defined and may vary among sRNA/mRNA pairs. This region of the sRNAs is evolutionarily conserved, often constituting the most conserved region of the molecule. Curiously, the seed region of many sRNAs is located in their 5'-ends, suggesting that position may impact function. Indeed, this is the case of SraL that interacts with *tig* mRNA through 10 nts located in its 5'-end. It was also shown for other sRNAs that the seed region can carry the regulatory function of the sRNA by itself and that it can perform the same regulatory effect when fused to other sequences (Papenfort *et al.*, 2010; Pfeiffer *et al.*, 2009). This suggests that sRNAs have active regions responsible for base-pairing with their targets, which can perform their functions without the context of the rest of the molecule.

During this Doctoral work we have revealed for the first time a biological function for SraL sRNA in *Salmonella* Typhimurium. However, taking into consideration the results obtained by proteomic analysis, there is a strong

indication of the existence of several other SraL targets in the cell. To further investigate this, we could use a pulse-expression approach, analysing the global changes of mRNA level after a transient overexpression of SraL from an inducible plasmid. The down- or up-regulations will suggest which mRNAs are directly regulated by the sRNA. SraL was shown to be highly expressed in SPI-2 inducing conditions and also in *S.* Typhimurium persisting inside eukaryotic cells (Ortega *et al.*, 2012; Viegas *et al.*, 2007). Therefore, one of the next steps will be the identification of functions that are targeted by SraL in *Salmonella* during the adaptation to an intracellular lifestyle inside the host eukaryotic cells. The transcriptomic analysis will be performed using samples obtained from both extracellular bacteria, grown under the SPI-2 inducing conditions, and intracellular bacteria.

In a previous work we have identified several RNases responsible for the post-transcriptional control of SraL sRNA. In this Dissertation we identified the *rpoS*-encoded σ^S subunit of RNA polymerase as a transcriptional regulator of SraL. We showed that RpoS binds directly to the promoter of SraL and governs its transcription. The members of the RpoS regulon are a diverse set of genes which functions are related to stress management, central metabolism, rearrangements of cell morphology, and virulence (Hengge-Aronis, 2002). Curiously, this sigma factor is post-transcriptionally regulated by several sRNAs. These sRNAs are synthesized in response to different stresses under the control of different regulators, allowing the bacterium to control RpoS translation by integrating the response to numerous stress signals (Battesti *et al.*, 2011). The fact that SraL sRNA has demonstrated to be highly regulated at both transcriptional and post-transcriptional levels reveals that it constitutes an important riboregulator in specific stress conditions that trigger RpoS expression. Furthermore, the importance of SraL is probably not exclusive to *Salmonella* Typhimurium since it is a very well conserved sRNA in several enteric bacteria. During this

Dissertation, it was only studied the biological role of SraL in late stationary phase of growth. It will be also of interest to study and understand the expression of this sRNA in other stress conditions known to trigger the RpoS-mediated response, such as UV-radiation, acid, temperature or osmotic shock and oxidative stress.

Along this Dissertation we have used *Salmonella* Typhimurium as a model organism. *Salmonella* is a Gram-negative and facultative intracellular pathogen that infects human and animals. Infections caused by *Salmonella* are a serious medical and veterinary problem worldwide, with an important incidence in the food industry since they are transmitted through the food chain (Mahan *et al.*, 1996). Given the emergence and prevalence of bacterial strains that are resistant to available antibiotics there is an increasing need for alternative approaches to anti-microbial therapy. Interestingly, it was recently reported the use of artificial *trans*-encoded sRNAs for specific gene silencing in bacteria (Man *et al.*, 2011). Thus, the study of the properties and functions of sRNAs in pathogenic bacteria can reveal important details that will contribute to the development of new strategies to combat infections by these bacteria.

RNases and sRNAs constitute important determinants in the regulation of gene expression. The study developed in this Dissertation revealed important information concerning the role of specific RNases and sRNAs in the post-transcriptional control in the human pathogen *Salmonella* Typhimurium.

References

Andrade, J.M., Pobre, V., Matos, A.M., and Arraiano, C.M. 2012. The crucial role of PNPase in the degradation of small RNAs that are not associated with Hfq. *RNA*. 18:844-855.

Arraiano, C.M., Andrade, J.M., Domingues, S., Guinote, I.B., Malecki, M., Matos, R.G., Moreira, R.N., Pobre, V., Reis, F.P., Saramago, M., Silva, I.J., and Viegas, S.C. 2010. The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol Rev*. 34:883-923.

Bartra, S.S., Gong, X., Lorica, C.D., Jain, C., Nair, M.K., Schifferli, D., Qian, L., Li, Z., Plano, G.V., and Schesser, K. 2012. The outer membrane protein A (OmpA) of *Yersinia pestis* promotes intracellular survival and virulence in mice. *Microb Pathog*. 52:41-46.

Battesti, A., Majdalani, N., and Gottesman, S. 2011. The RpoS-mediated general stress response in *Escherichia coli*. *Annu Rev Microbiol*. 65:189-213.

Bossi, L., and Figueroa-Bossi, N. 2007. A small RNA downregulates LamB maltoporin in *Salmonella*. *Mol Microbiol*. 65:799-810.

Busch, A., Richter, A.S., and Backofen, R. 2008. IntaRNA: efficient prediction of bacterial sRNA targets incorporating target site accessibility and seed regions. *Bioinformatics*. 24:2849-2856.

Coornaert, A., Lu, A., Mandin, P., Springer, M., Gottesman, S., and Guillier, M. 2010. MicA sRNA links the PhoP regulon to cell envelope stress. *Mol Microbiol*. 76:467-479.

Figueroa-Bossi, N., Lemire, S., Maloriol, D., Balbontin, R., Casadesus, J., and Bossi, L. 2006. Loss of Hfq activates the sigmaE-dependent envelope stress response in *Salmonella enterica*. *Mol Microbiol*. 62:838-852.

Fröhlich, K.S., Papenfort, K., Berger, A.A., and Vogel, J. 2012. A conserved RpoS-dependent small RNA controls the synthesis of major porin OmpD. *Nucleic Acids Res*. 40:3623-3640.

Gogol, E.B., Rhodius, V.A., Papenfort, K., Vogel, J., and Gross, C.A. 2011. Small RNAs endow a transcriptional activator with essential repressor functions for single-tier control of a global stress regulon. *Proc Natl Acad Sci U S A*. 108:12875-12880.

Görke, B., and Vogel, J. 2008. Noncoding RNA control of the making and breaking of sugars. *Genes Dev*. 22:2914-2925.

Han, X., Bai, H., Liu, L., Dong, H., Rui, L., Song, J., Ding, C., Qi, K., Liu, H., and Yu, S. 2012. The luxS gene functions in the pathogenesis of avian pathogenic *Escherichia coli*. *Microb Pathog*.

Hartl, F.U., and Hayer-Hartl, M. 2009. Converging concepts of protein folding *in vitro* and *in vivo*. *Nat Struct Mol Biol*. 16:574-581.

Hengge-Aronis, R. 2002. Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. *Microbiol Mol Biol Rev*. 66:373-395, table of contents.

Homerova, D., Rezuchova, B., Stevenson, A., Skovierova, H., Roberts, M., and Kormanec, J. 2011. Characterization of the *micA* gene encoding a small regulatory sigmaE-dependent RNA in *Salmonella enterica* serovar Typhimurium. *Folia Microbiol (Praha)*. 56:59-65.

Jepson, M.A., Collares-Buzato, C.B., Clark, M.A., Hirst, B.H., and Simmons, N.L. 1995. Rapid disruption of epithelial barrier function by *Salmonella typhimurium* is associated with structural modification of intercellular junctions. *Infect Immun*. 63:356-359.

Johansen, J., Rasmussen, A.A., Overgaard, M., and Valentin-Hansen, P. 2006. Conserved small non-coding RNAs that belong to the sigmaE regulon: role in down-regulation of outer membrane proteins. *J Mol Biol*. 364:1-8.

Kaberdin, V.R., and Blasi, U. 2006. Translation initiation and the fate of bacterial mRNAs. *FEMS Microbiol Rev*. 30:967-979.

Kawamoto, H., Koide, Y., Morita, T., and Aiba, H. 2006. Base-pairing requirement for RNA silencing by a bacterial small RNA and acceleration of duplex formation by Hfq. *Mol Microbiol*. 61:1013-1022.

Kint, G., De Coster, D., Marchal, K., Vanderleyden, J., and De Keersmaecker, S.C. 2010. The small regulatory RNA molecule MicA is involved in *Salmonella enterica* serovar Typhimurium biofilm formation. *BMC Microbiol*. 10:276.

Kramer, G., Boehringer, D., Ban, N., and Bukau, B. 2009. The ribosome as a platform for co-translational processing, folding and targeting of newly synthesized proteins. *Nat Struct Mol Biol*. 16:589-597.

Lasa, I., Toledo-Arana, A., and Gingeras, T.R. 2012. An effort to make sense of antisense transcription in bacteria. *RNA Biol*. 9.

Lee, J.S., Jung, I.D., Lee, C.M., Park, J.W., Chun, S.H., Jeong, S.K., Ha, T.K., Shin, Y.K., Kim, D.J., and Park, Y.M. 2010. Outer membrane protein a of *Salmonella enterica* serovar Typhimurium activates dendritic cells and enhances Th1 polarization. *BMC Microbiol*. 10:263.

Mahan, M.J., Slauch, J.M., and Mekalanos, J.J. 1996. Environmental regulation of virulence gene expression in *Escherichia*, *Salmonella* and *Shigella* spp. *In E. coli* and *Salmonella:* Cell and Molecular Biology. F.C. Neidhardt, R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, and H.E. Umbarger, editors. American Society for Microbiology, Washington, D.C. 2803-2815.

Man, S., Cheng, R., Miao, C., Gong, Q., Gu, Y., Lu, X., Han, F., and Yu, W. 2011. Artificial *trans*-encoded small non-coding RNAs specifically silence the selected gene expression in bacteria. *Nucleic Acids Res*. 39:e50.

Massé, E., Majdalani, N., and Gottesman, S. 2003. Regulatory roles for small RNAs in bacteria. *Curr Opin Microbiol*. 6:120-124.

Muckstein, U., Tafer, H., Hackermuller, J., Bernhart, S.H., Stadler, P.F., and Hofacker, I.L. 2006. Thermodynamics of RNA-RNA binding. *Bioinformatics*. 22:1177-1182.

Ortega, A.D., Gonzalo-Asensio, J., and García-Del Portillo, F. 2012. Dynamics of *Salmonella* small RNA expression in non-growing bacteria located inside eukaryotic cells. *RNA Biol*. 9.

Papenfort, K., Bouvier, M., Mika, F., Sharma, C.M., and Vogel, J. 2010. Evidence for an autonomous 5' target recognition domain in an Hfq-associated small RNA. *Proc Natl Acad Sci U S A*. 107:20435-20440.

Papenfort, K., Pfeiffer, V., Mika, F., Lucchini, S., Hinton, J.C., and Vogel, J. 2006. SigmaE-dependent small RNAs of *Salmonella* respond to membrane stress by accelerating global *omp* mRNA decay. *Mol Microbiol*. 62:1674-1688.

Pfeiffer, V., Papenfort, K., Lucchini, S., Hinton, J.C., and Vogel, J. 2009. Coding sequence targeting by MicC RNA reveals bacterial mRNA silencing downstream of translational initiation. *Nat Struct Mol Biol*. 16:840-846.

Preissler, S., and Deuerling, E. 2012. Ribosome-associated chaperones as key players in proteostasis. *Trends Biochem Sci*. 37:274-283.

Sittka, A., Lucchini, S., Papenfort, K., Sharma, C.M., Rolle, K., Binnewies, T.T., Hinton, J.C., and Vogel, J. 2008. Deep sequencing analysis of small noncoding RNA and mRNA targets of the global post-transcriptional regulator, Hfq. *PLoS Genet*. 4:e1000163.

Tjaden, B. 2008. TargetRNA: a tool for predicting targets of small RNA action in bacteria. *Nucleic Acids Res*. 36:W109-113.

Udekwu, K.I. 2010. Transcriptional and post-transcriptional regulation of the *Escherichia coli luxS* mRNA; involvement of the sRNA MicA. *PLoS One*. 5:e13449.

Udekwu, K.I., Darfeuille, F., Vogel, J., Reimegard, J., Holmqvist, E., and Wagner, E.G. 2005. Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. *Genes Dev*. 19:2355-2366.

Udekwu, K.I., and Wagner, E.G. 2007. Sigma E controls biogenesis of the antisense RNA MicA. *Nucleic Acids Res*. 35:1279-1288.

Vanderpool, C.K., Balasubramanian, D., and Lloyd, C.R. 2011. Dual-function RNA regulators in bacteria. *Biochimie*. 93:1943-1949.

Viegas, S.C., Pfeiffer, V., Sittka, A., Silva, I.J., Vogel, J., and Arraiano, C.M. 2007. Characterization of the role of ribonucleases in *Salmonella* small RNA decay. *Nucleic Acids Res*. 35:7651-7664.

Wu, H.H., Yang, Y.Y., Hsieh, W.S., Lee, C.H., Leu, S.J., and Chen, M.R. 2009. OmpA is the critical component for *Escherichia coli* invasion-induced astrocyte activation. *J Neuropathol Exp Neurol*. 68:677-690.

Zhang, Y., Sun, S., Wu, T., Wang, J., Liu, C., Chen, L., Zhu, X., Zhao, Y., Zhang, Z., Shi, B., Lu, H., and Chen, R. 2006. Identifying Hfq-binding small RNA targets in *Escherichia coli*. *Biochem Biophys Res Commun*. 343:950-955.

Appendix

PUBLICATIONS

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Characterization of the role of ribonucleases in *Salmonella* small RNA decay

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ABSTRACT

In pathogenic bacteria, a large number of sRNAs coordinate adaptation to stress and expression of virulence genes. To better understand the turnover of regulatory sRNAs in the model pathogen, *Salmonella typhimurium*, we have constructed mutants for several ribonucleases (RNase E, RNase G, RNase III, PNPase) and Poly(A) Polymerase I. The expression profiles of four sRNAs conserved among many enterobacteria, CsrB, CsrC, MicA and SraL, were analysed and the processing and stability of these sRNAs was studied in the constructed strains. The degradosome was a common feature involved in the turnover of these four sRNAs. PAPI-mediated polyadenylation was the major factor governing SraL degradation. RNase III was revealed to strongly affect MicA decay. PNPase was shown to be important in the decay of these four sRNAs. The stability of CsrB and CsrC seemed to be independent of the RNA chaperone, Hfq, whereas the decay of SraL and MicA was Hfq-dependent. Taken together, the results of this study provide initial insight into the mechanisms of sRNA decay in *Salmonella*, and indicate specific contributions of the RNA decay machinery components to the turnover of individual sRNAs.

INTRODUCTION

Regulatory mechanisms involving small untranslated RNAs (sRNAs) have received considerable attention over the past decade. Eukaryotic and prokaryotic cells contain a wealth of these regulators with determinant roles in the post-transcriptional control of gene expression. To date, a variety of experimental and computational approaches have identified close to hundred sRNA genes in *Escherichia coli* K12 (1–3), many of which are conserved in diverse enteric bacteria, including pathogenic *Salmonella* species (4).

The mechanisms by which sRNAs modulate gene expression are diverse, and two general modes of action have been established, dividing regulatory RNAs into two classes (5). The sRNAs belonging to the first class act by interaction with a protein to modify its activity. The other class consists of sRNAs that act by base pairing with one or more target mRNAs. Most of these antisense RNAs act with partial complementarity over *trans*-encoded target mRNAs to modify their translation and/or stability. Such *trans*-sRNAs typically require the bacterial RNA chaperone, Hfq, both for target interaction and for intracellular stability. It is generally assumed that Hfq binds both the regulator and the target RNA, favouring their interaction. Hfq enhances the stability of many sRNAs *in vivo*, by protecting them from degradation (6–10).

To understand the action of regulatory sRNAs, it is also fundamental to study the processing and turnover of these molecules. Previous work in *Escherichia coli* and other bacteria established that the sRNAs differ greatly in stability, what is probably related with their biological function; some are very stable with long half-lives whilst others are turned over within few minutes (6,11). Since ribonucleases (RNases) are key modulators of RNA decay, the identification of the RNases that contribute to the decay of individual sRNAs is essential for a more general understanding of sRNA turnover *in vivo*.

In *E. coli*, the main endoribonucleases are RNase E, RNase G and RNase III (12,13). RNase E is a single-stranded-specific endoribonuclease with a main role in *E. coli* mRNA decay, being also involved in the processing of ribosomal and transfer RNAs. RNase E is also one of the main enzymes forming the degradosome, a multiprotein complex involved in the decay of many RNAs (14,15). RNase G (also known as CafA protein), was shown to be a homologue of the N-terminal catalytic domain of RNase E (16,17). This endoribonuclease is involved in the 5′ end-processing of 16S rRNA and also in

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Table 1. List of strains and plasmids used in this work

Strain	Relevant Markers/Genotype	Source/Reference
S. typhimurium, SL1344	Str^R*hisG rpsL xyl*	(87), provided by Dirk Bumann, MPI-IB Berlin
CMA-537	SL1344 *rne-537* (Δ*rne*::Cm^R)	This study
CMA-539	SL1344 *pnp-539* (Δ*pnp*::Cm^R)	This study
CMA-542	SL1344 *pcnB-542* (Δ*pcnB*::Cm^R)	This study
CMA-550	SL1344 *rng-550* (Δ*rng*::Cm^R)	This study
CMA-555	SL1344 *ompA-555* (Δ*ompA*::Cm^R)	This study
JVS-938	SL1344 *rnc-938* (Δ*rnc*::Kan^R)	This study
JVS-00255	SL1344 Δ*hfq*::Cm^R	(42)
JVS-00067	SL1344 Δ*csrB*::Kan^R	This study[a]
JVS-00084	SL1344 Δ*csrC*::Kan^R	This study[a]
E. coli DH5α	*rec*A1 *end*A1 *gyr*A96 thi-*hsd*R17 *sup*E44 *rel*A1 Δ*lac*ZYA-*arg*FU169 f80dLacZDM15	New England Biolabs

Plasmid	Comment	Origin/Marker	Reference
pSVA-5	IPTG inducible plasmid expressing PNPase	pSE420/Amp^R	This study
pKD3	Template for mutants construction; carries chloramphenicol-resistance cassette	oriRγ/Amp^R	(40)
pKD4	Template for mutants construction; carries kanamycin-resistance cassette	oriRγ/Amp^R	(40)
pKD46	Red Helper Plasmid	Amp^R	(40)

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mRNA degradation in *E. coli*. Both RNase E and RNase G cleave single-stranded regions of structured RNAs, and share a preference for 5′ monophosphate termini and AU-rich sequences of RNA (18). RNase III is specific for double-stranded RNA and plays multiple roles in the processing of rRNA and mRNA (19). This enzyme can also affect the decay of some messages (20,21). Exoribonucleases are enzymes that degrade RNA from its extremity (13,22–25). PNPase, one of the main exoribonucleases, is widespread both in the eubacteria and eukaryotes and associates with RNase E in the degradosome (15). Poly(A) polymerase I (PAP I) can also modulate RNA stability by adding poly(A) tails to the 3′ end of RNAs (26–29). The Poly(A) tail provides a 'toehold' for the efficient exonucleolytic degradation of the RNA (especially if this RNA is structured). PAP I can be a main factor involved in mRNA decay and affects other processes such as transcription and proteolysis (30,31).

In this work we report the construction, in the pathogen *Salmonella typhimurium*, of mutant strains for RNase E, G, III, PNPase and PAP I. We have investigated the effects of these mutants on the accumulation and turnover of four regulatory sRNAs of *Salmonella* (CsrB, CsrC, MicA and SraL). CsrB and CsrC are an example of regulatory RNAs that interact with a protein. Together with the RNA-binding protein CsrA, they form the Csr (Carbon Storage Regulator) complex, one of the key regulatory circuits of virulence in *Salmonella* (32,33). CsrB and CsrC sRNAs have similar structures with multiple stem-loops that sequester several CsrA proteins impairing their interaction with the targets (34). MicA sRNA is expressed in numerous enterobacteria (35), and has been shown to repress the *trans*-encoded *ompA* and *lamB* porin mRNAs in *E. coli* and *Salmonella* (7,35,36). Outer membrane protein A (OmpA) was the first and most studied MicA target. *ompA* mRNA levels decrease upon entry into stationary phase (7,35,37), concomitantly with MicA accumulation. MicA binds to *ompA* mRNA translation initiation region (TIR) interfering with ribosome binding (35), which most likely renders the mRNA more accessible to endonucleolytic cleavage. SraL sRNA was previously described in *E. coli* (38,39), and *sraL* genes have been predicted in several enteric bacteria (4). However, SraL function and target(s) have yet to be elucidated.

The results obtained in this work give relevant information about the expression of these four sRNAs in *Salmonella* and identify some of the main enzymes that are involved in their turnover, bringing initial insight into the underlying mechanisms of sRNA decay in this bacterial model organism.

MATERIALS AND METHODS

Bacterial strains and plasmids

All *Salmonella* strains used in this study are isogenic derivatives of the wild-type *Salmonella enterica* serovar Typhimurium strain SL1344. Strains and Plasmids used in this study are listed in Table 1. The RNase mutants were constructed following the lambda-red recombinase method (40), with few modifications. The strain carrying plasmid pKD46 was grown in SOC with ampicillin and 0.2% L-arabinose at 28°C to an OD_{600} of 0.5 and then made electrocompetent by successive washings in ice-cold water and concentrating 400-fold in ice-cold 10% glycerol. To construct the deletion strains, the *cat* chloramphenicol-resistance gene was amplified from plasmid pKD3 with oligonucleotides carrying ~50 bp-homology extensions to the respective target genes. For the construction of RNase III$^-$ mutant (JVS-938 strain) the Kan-resistance cassette was amplified from pKD4 plasmid. Fifty microlitres of competent cells were mixed with the purified PCR product

(~100 ng) in a chilled cuvette (0.2 cm electrode gap) and electroporated (18 kV cm^{-1}). Subsequently, 1 ml of pre-warmed SOC medium was added, and cells were recovered after incubation for 1 h at 37°C before selection on LB agar plates with the appropriate antibiotics. All mutations were moved to a fresh SL1344 background by P22 HT105/1 int-201 transduction (41).

The mutant strains were constructed as shown in Figure S1 of Supplementary Data. All gene deletions were verified by PCR. C-terminal truncation of RNase E in CMA-537 was verified by PCR and western blot using an *E. coli* RNase E antiserum that cross-reacts with *Salmonella* homologue (kindly provided by A. J. Carpousis).

For construction of pSVA-5 plasmid (Table 1) expressing PNPase, a PCR fragment containing the entire *pnp* sequence was amplified from SL1344 chromosome and was cloned into the XbaI and EcoRI sites of the IPTG inducible plasmid pSE420 (Invitrogen). Competent *E. coli* DH5α cells (New England Biolabs) were used for cloning procedures during plasmid construction.

Bacterial growth

All strains were grown in Luria–Bertani (LB) broth at 37°C and 220 r.p.m. throughout this study, unless stated otherwise. SOC medium was used to recover cells after transformation. Electroporation and heat-shock procedures were used for transformation of *Salmonella* and *E. coli*, respectively. M9 was used for experiments with minimal medium. Conditions indicated as 'SPI-1 and SPI-2 inducing conditions' corresponded to growth in high salt medium (0.3 M NaCl) with low oxygen in sealed Falcon tubes, as described for SPI-1 induction (42), and in PCN medium (1 mM phosphate, pH 5.8) as described for SPI-2 induction (43).

Growth medium was supplemented with the following antibiotics where appropriate: ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml) and streptomycin (90 μg/ml).

RNA extraction and northern blot analysis

Overnight cultures were diluted 1/100 in fresh medium and grown to the indicated cell densities at OD_{600} (growth medium and conditions are detailed in the respective figure legends). Culture samples were collected, mixed with 0.2 volume of stop solution (5% water-saturated phenol, 95% ethanol), and frozen in liquid nitrogen. After thawing on ice, bacteria were pelleted by centrifugation (2 min, 16 000 r.c.f., 4°C), and RNA was isolated using the Trizol method (Invitrogen) following the manufacture's instructions. For stability experiments, rifampicin (500 mg ml^{-1}) and nalidixic acid (20 mg ml^{-1}) were added to cells grown in LB at 37°C, 220 r.p.m., till OD2 and/or 6 h after. Incubation was continued and culture aliquots were withdrawn at the times indicated in the respective figures. RNA was extracted, visualized on agarose gel and then quantified on a Nanodrop machine (NanoDrop Technologies).

For northern blot analysis, RNA samples were denatured for 10 min at 80°C in RNA loading buffer (95% [v/v] formamide, 0.1% [w/v] xylene cyanol, 0.1% [w/v] bromophenol blue, 10 mM EDTA), separated on 8.3 M urea/6% polyacrylamide gels, and transferred to Hybond-XL membranes (GE Healthcare) by electroblotting (1 h, 50 V, 4°C) in a tank blotter (Peqlab, Germany). Following pre-hybridization of the membranes in Rapid-hyb Buffer (GE Healthcare), membranes were hybridized at 70°C with riboprobes, or at 42°C in the case of oligoprobes. After hybridization, membranes were rinsed at room temperature in a 2× SSC/0.1% SDS solution, followed by washing in three subsequent 15 min steps in SSC (2×, 1× or 0.5×, respectively)/0.1% SDS solutions at the hybridization temperature. Membranes hybridized with the oligoprobes were rinsed in 5× SSC/0.1% SDS solution followed by three wash steps at 42°C in SSC (5×, 1× and 0.5×, respectively)/0.1% SDS solutions. Signals were visualized on a Phosphorimager (FLA-3000 Series, Fuji), and band intensities quantified with AIDA software (Raytest, Germany).

Primer extension analysis

Total RNA was extracted as described above. Primers CsrC-II and CsrC-IV are complementary to CsrC in positions +37 to +57 and +151 to +170, respectively, (+1 corresponds to RNA start site). Primer CsrB-III is complementary CsrB in positions +302 to +321 relative to CsrB start site. Primers were end-labelled using T4 polynucleotide kinase and [-^{32}P]ATP (Fermentas). Unincorporated [^{32}P]-γ-ATP was removed using a MicroSpinTM G-25 Column (GE Healthcare). A total of 2 pmol of primer was annealed to 10 μg of RNA and cDNA was synthesized using 200 U of Superscript III RT from Invitrogen. The same labelled primer was used to generate a corresponding DNA sequencing ladder using the Cycle Reader DNA Sequencing Kit (Fermentas). The PCR fragment used as template for the sequencing reaction was amplified from SL1344 strain with primers CsrC-IV and CsrC-seq for CsrC and CsrB-III and CsrB-seq for CsrB. The primer extension products were separated in parallel with the sequencing ladder on a 6% polyacrylamide sequencing gel containing 7 M urea. The gel was dried and exposed. Signals were visualized in a PhosphorImager (Storm Gel and Blot Imaging system, Amersham Bioscience).

Hybridization probes

Primers for template amplification are listed in Table S1 (Supplementary Data). Standard polymerase chain reactions were carried out on genomic DNA. Riboprobes were generated from PCR fragments (a T7 RNA polymerase promoter sequence was added by the antisense primer) in the presence of an excess of [^{32}P]-α-UTP over unlabelled UTP using the Ambion T7 polymerase Maxiscript kit. DNA oligonucleotides were labelled with [^{32}P]-γ-ATP using T4 polynucleotide kinase (Fermentas). All labelled probes were purified over G50

columns (GE Healthcare) to remove unincorporated nucleotides prior to hybridization.

RESULTS

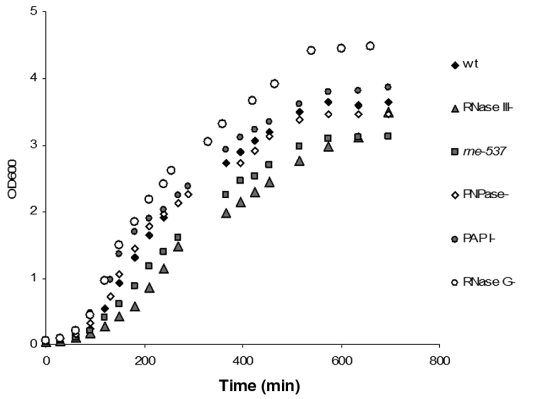
Construction and characterization of *Salmonella* RNase mutant strains

All RNase mutants (listed in Table 1) were constructed in the virulent *Salmonella typhimurium* strain, SL1344. The sequences of the genomic regions of interest, taken from the unfinished genome of SL1344 (http://www.sanger.ac.uk/Projects/Salmonella), were compared with that of the sequenced *Salmonella* strain LT2 (44) and found to be identical.

Our strategy was to create *Salmonella* mutants similar to those that have been characterized in *E. coli* (45–47). The RNase gene sequences of both bacteria were compared in terms of amino acids and nucleotide sequences in order to create equivalent gene deletions. Deletion/substitution mutants were constructed through the replacement of part of the coding sequence by a resistance marker (for details see Materials and Methods section and Figure S1 in the Supplementary Data). For RNase E, encoded by an essential gene (*rne*), we have constructed a mutant, which is deleted for the C-terminal scaffold of the enzyme (*rne-537* mutation). This is the region responsible for the protein–protein interactions in the formation of the ribonucleolytical complex called degradosome (48). A similar mutant exists in *E. coli* (*rne-131* mutation). This mutant was reported to stabilize mRNAs, leaving rRNA processing unaffected (49). The mutant is defective in both the interaction with the chaperon Hfq, and the assembly of a functional degradosome (9,50).

Loss of RNase III function in the RNase III$^-$ insertion mutant was confirmed by a specific defect in rRNA processing. That is, the absence of a functional RNase III impairs rRNA processing in both *E. coli* and *Salmonella* (51,52). In *Salmonella typhimurium*, RNase III promotes the excision of intervening sequences (IVSs) causing the fragmentation of 23S rRNA (52), which we observed to be abrogated in the RNase III$^-$ mutant strain constructed here (data not shown).

We have compared the growth properties of the wild-type SL1344 with RNase and PAP I mutant strains grown in Luria broth at 37°C (Figure 1). For the majority of the mutants, the lag period necessary for recovery from stationary phase was comparable to the wild-type strain. Loss of RNase III resulted in the slowest growth rate (Figure 1); the generation time of the RNase III$^-$ mutant doubled in comparison to the wild-type strain. We note that this growth defect was even more severe on plates since this strain took ~24–36 h to form colonies of the size formed by the wild-type strain overnight. The RNase E and PNPase mutations also affected growth rate, causing a slower growth. Namely, *rne*-537 mutant had a longer generation time (41 min) than the wild type (30 min). Regarding the RNase G$^-$ deficient strain, albeit the generation time was not significantly different, the strain reached a considerably higher cell density in stationary phase.



Strain	wt	RNase III$^-$	*rne-537*	PNPase$^-$	PAP I$^-$	RNase G$^-$
Generation time (min)	30±3	59±5	41±4	37±6	32±5	33±2

Figure 1. Comparison of growth profiles of wild-type, RNase III, E, G, PNPase and PAP I mutant strains. Strains were grown in LB medium at 37°C. The values of generation time are the result of at least three independent growth curves.

Analysis of sRNA expression under different growth conditions

Many of the sRNAs previously characterized in *E. coli* K12 are induced under specific stress conditions, e.g. upon oxidative stress (53), DNA damage (54), cold shock (55), iron stress (56) and osmotic stress (57). However, the steady-state levels of many of such sRNAs are also increased in stationary phase (11,38,58). Therefore, we first analysed the expression of the four sRNAs selected here in wild-type cells at different phases of growth in LB and minimal media, in order to determine conditions in which we could study their processing and decay. We also included two growth conditions known to induce the two major *Salmonella* virulence regions, i.e. the *Salmonella* Pathogenicity Islands (SPI) 1 and 2. The virulence genes encoded by SPI-1 facilitate the entry of *Salmonella* into non-phagocytic cells. SPI-1 genes are specifically expressed in early stationary phase cultures of *Salmonella* grown in standard LB medium (59), and are also highly induced by oxygen tension and elevated osmolarity (60). The genes of SPI-2 encode virulence factors for intra-macrophage survival and systemic disease; these genes are upregulated, *in vitro*, in minimal media with low phosphate and magnesium concentrations (43).

The CsrB (363 nt) and CsrC (244 nt) RNAs highly accumulated upon entry into stationary phase (in LB) and under SPI-1 inducing conditions (Figure 2). This pattern was in agreement with previous observations that the Csr system represses a variety of stationary-phase genes, and that the loss of both CsrB and CsrC significantly reduces SPI-1 gene expression and epithelial cell invasion (33). The blots shown in Figure 2 also indicate that these two sRNAs are not expressed under SPI-2 inducing

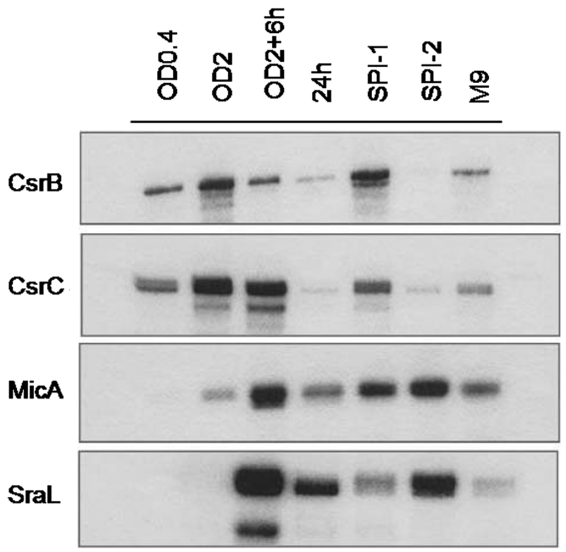


Figure 2. Analysis of sRNA expression under different growth conditions. Northern blot analysis of sRNA expression in *Salmonella* SL1344 grown under different conditions as indicated in figure labels. Cells were grown in LB at 37°C till OD_{600} of 0.4 (OD0.4), 2 (OD2), 6 h after (OD2 + 6 h) and for twenty-four hours (24 h). Cells were also grown under conditions of induction of pathogenicity islands 1 and 2 (SPI-1, SPI-2) and in minimal medium till an OD_{600} of 2 (M9). A total of 2.5 µg of RNA were run on a 6% PAA/8.3 M urea gel, blotted and probed as described in Material and Methods section.

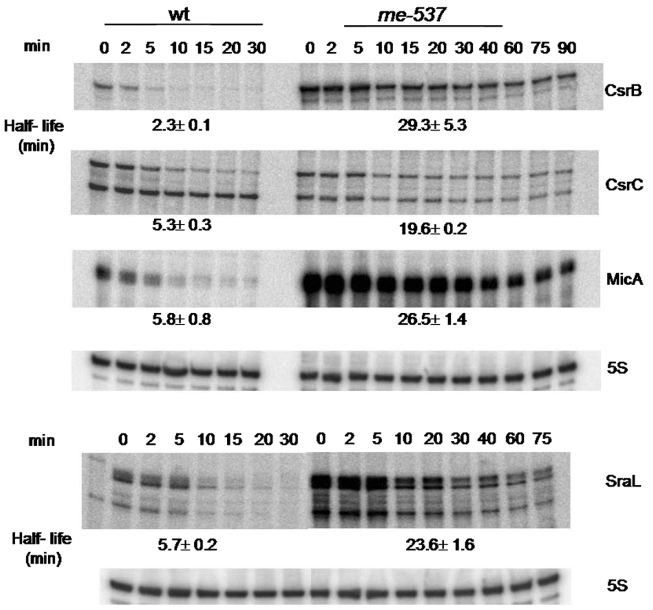


Figure 3. RNase E mutation strongly affects sRNA stability. Northern blot analysis of the stability of CsrB, CsrC, MicA and SraL transcripts in wild- type and *rne-537* mutant. RNA was extracted from bacteria grown in LB medium at 37°C, till 6 h after OD_{600} 2. At this time, a mixture of rifampicin and nalidixic acid was added to growing cells and samples were removed at the times indicated. Total RNA was extracted and 20 µg of RNA (each lane) was separated on a 6%PAA/8.3M urea gel. The gel was then blotted to a *Hybond-N+* membrane and hybridized with the corresponding sRNA riboprobe. Details of RNA extraction and 'northern blot' procedure are described in Materials and Methods section. The three first panels (CsrB, CsrC, MicA) correspond to the same membrane that was hybridized with each of the sRNA probes indicated. SraL was hybridized with another membrane. In each case, the membrane was stripped and then probed for 5S RNA as loading control. The band corresponding to the full-length transcript was quantified and plotted versus time of extraction (in minutes) to calculate the half-life of the sRNA. A representative membrane is shown and the half-life values indicated correspond to the average of several 'northern blot' experiments with RNAs from at least two independent extractions.

conditions, i.e. when the genes necessary for proliferation of *Salmonella* in macrophages are transcribed.

The ~74 nt sRNA MicA became detectable at early stationary phase of growth in LB medium, and strongly accumulated when growth further slowed down (Figure 2), as shown previously (61,62). Interestingly, MicA levels under SPI-1 and SPI-2 inducing conditions were comparable to those in stationary phase.

SraL (140 nt) was not detectable before the cells reached stationary phase (Figure 2), which is fully in line with the late stationary phase-specific expression of SraL in *E. coli* (38,58). For all the four sRNAs studied, the expression was low at twenty-four hours of growth (24 h) as compared to the OD2 + 6 h condition. Interestingly, there was no substantial accumulation of these sRNAs in minimal medium (M9), even though growth in minimal medium constitutes a stress for the cell. Note that few *E. coli* sRNAs have a high expression under this condition (11,38,58).

In summary, all four sRNAs were significantly expressed at OD2 + 6 h, which we have chosen as the 'consensus' condition to subsequently study their decay in rifampicin-treatment experiments.

Degradosome is a major factor in sRNA turnover in *Salmonella*

RNase E is the enzyme that serves as the scaffold for the other protein components in the degradosome assembly. The absence of degradosome assembly (C-terminal truncation in *rne*-537 mutant) caused a large stabilization of all four sRNAs studied in this work. Notably, for CsrB, CsrC and MicA the absence of a full-length RNase E had the strongest stabilization effect in comparison to the other RNase mutants investigated here. Figure 3 shows that CsrB was highly stabilized (>12-fold) in this mutant since it decayed with a half-life of ~29 min as compared to ~2 min in the wild type. The other CsrA-antagonist, CsrC sRNA, was stabilized ~4-fold. The CsrB and CsrC sRNA decay was not strictly logarithmic; it was biphasic. The fact that both sRNAs are highly structured, i.e. CsrB and CsrC contain 16 and 8 stem-loops respectively, may help explain this behaviour (32,33). Their decay is very fast at the first minutes but at the second phase it is very slow, which may be due to the occurrence of highly stable intermediates during the decay. Since it was difficult to determine an exact half-life we have chosen to compare the stability of the sRNAs over the stage where the decay is still logarithmic. Thus, in wild-type *Salmonella*, the half-lives of CsrB and CsrC are ~2 min and ~5 min, respectively (Figure 3).

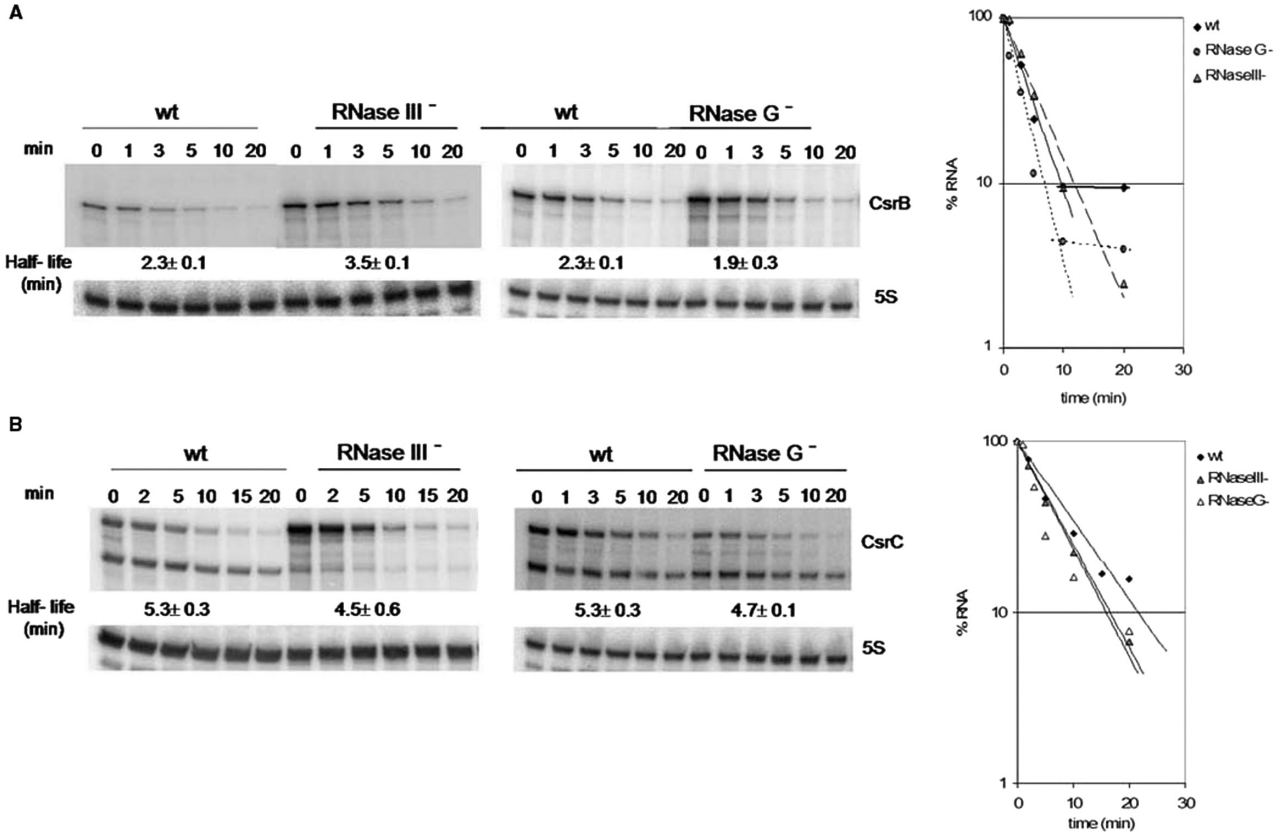


Figure 4. Endoribonuclease III and G do not significantly affect CsrB and CsrC turnover rates. Analysis of (**A**) CsrB and (**B**) CsrC decay in the absence of RNase III and RNase G. The experimental procedure was similar to the one described in Figure 3. The same membrane was, in each case, probed for 5S RNA as loading control. A representative membrane is shown and the half-life values correspond to the average of several 'northern blot' experiments with RNAs from at least two independent extractions.

The impairment of degradosome formation also impacted on MicA decay, with a ~5-fold stabilization of the transcript. Similarly, SraL transcripts were also significantly stabilized in this mutant, i.e. ~4-fold (Figure 3).

Due to the substantial effects of *rne-537* mutant on the decay of these sRNAs, we also investigated the effects of two additional major endoribonucleases, RNase III and G. Neither mutant substantially affected CsrB or CsrC stability (Figure 4). However, whereas CsrC transcript possesses two bands in the wild type, in the RNase III mutant the larger band (~240 nt) is the most prominent, which probably means that the ribonuclease has a role in the processing of this sRNA. Regarding MicA, RNase G does not seem to be involved in this sRNA decay under the growth condition assayed here. However, the loss of RNase III activity rendered this sRNA exceptionally stable (Figure 5A).

PNPase absence affects sRNA turnover in different ways

PNPase is the other ribonuclease component of the degradosome. We have also investigated the effects of the loss of this enzyme. Absence of PNPase had a large effect on MicA stability, causing a ~3.3-fold increase in MicA half-life (Figure 5B). However, this stabilization effect was slightly less than the one obtained in the absence of degradosome assembly (~5-fold). SraL sRNA was stabilized to a similar degree in the absence of PNPase and in the *rne*-537 mutant (~3- and ~4-fold, respectively; Figure 9A). Moreover, both mutants resulted in a similar SraL RNA pattern (see below). In contrast, absence of PNPase resulted in a CsrB RNA pattern entirely different from the wild-type strain. Specifically, several decay intermediates became observable, which were not detected in the wild-type strain. Since the growth rate can affect the expression and processing of sRNAs, we tested whether this alteration was maintained in another growth condition. In standard media (LB), CsrB is most highly expressed in early stationary phase (OD_{600} of 2, Figure 2). The same CsrB degradation pattern in *pnp* mutant was obtained at both growth conditions (Figure 6A). Regarding CsrC sRNA, the pattern of the bands was also changed in the PNPase$^-$ strain (Figure 6B). Complementation of PNPase$^-$, by providing *pnp* in *trans* from a plasmid, restored both CsrB and CsrC degradation pattern to the wild-type characteristics (Figure 6).

In order to analyse the origin of this different decay pattern for CsrB and CsrC in some of the mutant strains analysed, we have mapped the 5′ end of the corresponding breakdown products in these strains. For CsrC, we have

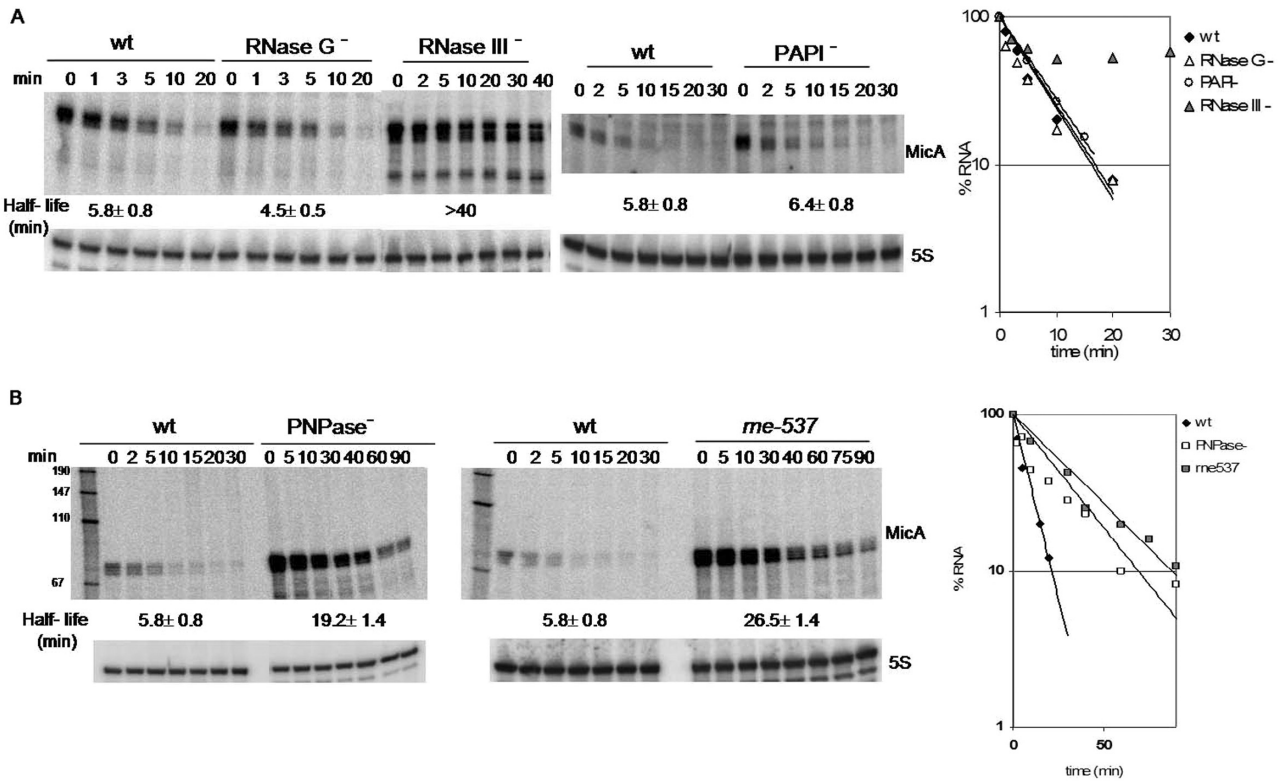


Figure 5. Analysis of MicA turnover. (**A**) Comparison of the effects of endoribonucleases G and III and PAP I in MicA stability. (**B**) RNase E and PNPase mutations highly affect the stability of MicA sRNA. The experimental procedure was similar to the one described in Figure 3. The same membrane was, in each case, probed for 5S RNA as loading control. A representative membrane is shown and the half-life values correspond to the average of several 'northern blot' experiments with RNAs from at least two independent extractions.

used two distinct primers along the sRNA, one located close to the terminator and the other binding at the middle of the sRNA (primers II and IV, respectively, in Figure 7A). In all the strains analysed, primer extension analysis of CsrC yielded a unique extension product that corresponded to the 5′ end of full-length CsrC RNA (Figure 7C). Interestingly, the intensity of the primer extension signal obtained with primer IV varies among the strains analysed. In strain RNase III$^-$, the intensity of the larger fragment is much higher than in wild type and PNPase$^-$. This is in full agreement to what is seen in the northern blot (Figure 7B, full-length probe). This must be due to the fact that the shorter fragment has a different 3′ end at which primer IV (near the end of the sRNA) cannot anneal. Therefore, the extension product in this reaction corresponds only to the larger fragment as opposed to what is observed with primer II, which detects both the large and the short fragment (Figure 7C). Therefore, we have done northern blot analysis of the two sRNAs using different probes along the two genes, to confirm these predictions. In the case of CsrC, the hybridization of the sRNA with a riboprobe encompassing the entire gene gives two major products in the wild type (Figure 7B, full-length probe). The shorter fragment is dependent of RNase III and accumulates in PNPase$^-$ strain. When using the primer IV, located near the terminator of the sRNA (Figure 7A), the larger band was the only fragment detectable. This seems to result from the fact that the shorter band has a different 3′ end and does not anneal with primer IV. With primers I, II and III this shorter band is detected. The results from northern blot analysis confirmed primer extension results and showed that the intermediary fragments have the same 5′ ends but different 3′ ends.

The CsrB primer located near the terminator (primer III in Figure 8A) also gave a unique band in the primer extension (Figure 8C). The 5′ end mapped to the +1 site of CsrB in *Salmonella*. The northern blot analysis using different probes along the CsrB RNA sequence has also revealed that the intermediary bands that accumulate in PNPase$^-$ strain have different 3′ ends. When using primer III near the terminator, we were only able to see the band corresponding to full-length CsrB. With primer II, annealing between nts 210 and 240 we were able to detect the full-length band and the band corresponding to ~240 nt (Figure 8B). Primer I, annealing approximately between the 160 and 180 nt, gave the same band pattern as with a probe directed against the entire CsrB RNA (Figure 8B).

Polyadenylation as a determinant factor in SraL decay

Our analysis of SraL decay in several RNase mutants and the PAP I$^-$ mutant revealed several differences with respect to the wild type. First, SraL is highly stabilized

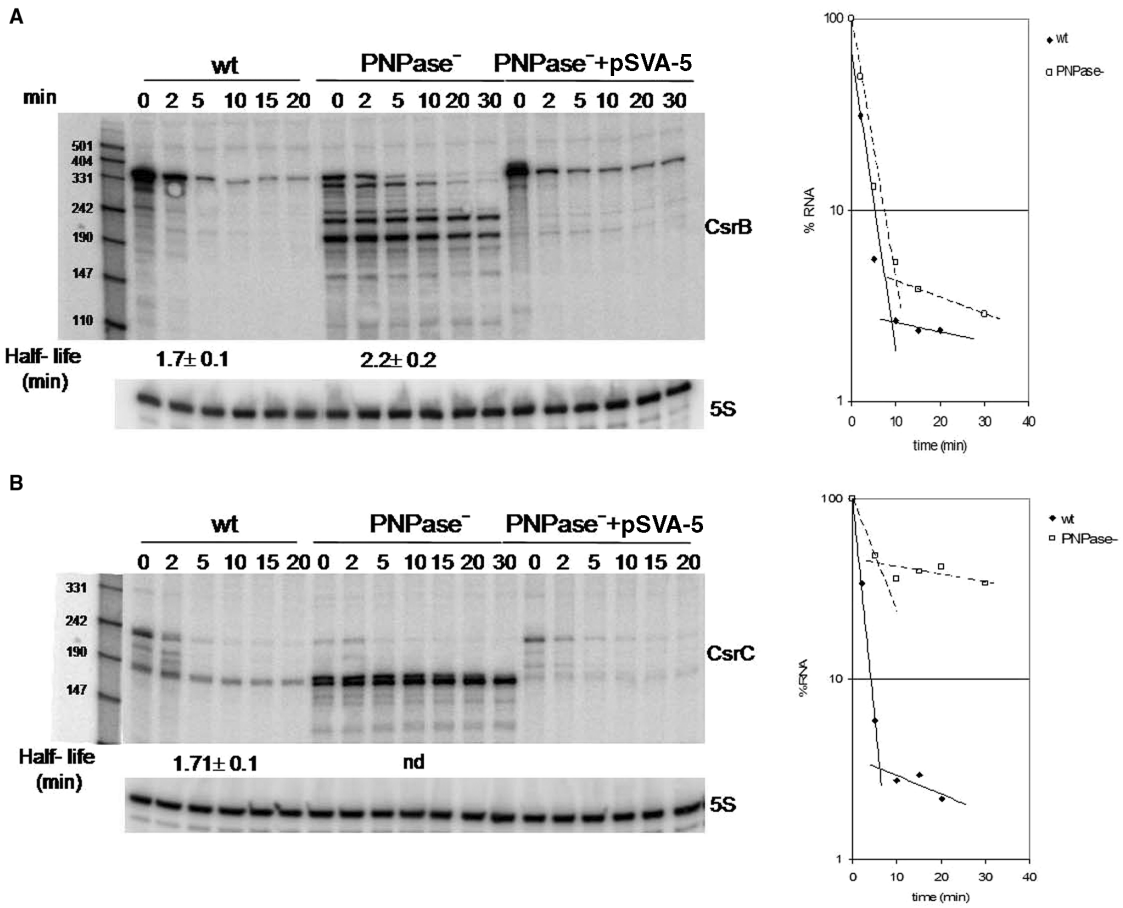


Figure 6. Analysis of PNPase effect on CsrB and CsrC decay. Northern blot analysis of (**A**) CsrB and (**B**) CsrC transcripts decay in wild-type, PNPase⁻ mutant and a strain where the mutation was complemented with a plasmid overexpressing PNPase. Strains were grown until 6 h after OD2. The value of CsrC half-life in PNPase⁻ mutant was not determined (nd) since the degradation of the transcript is immediately stabilized after the first five minutes of decay. In the first phase of the curve, the transcript decay rate is not significantly different from the wild type. Procedures in both cases were essentially as described in Figure 3. In each case, the membrane was stripped and then probed for 5S RNA as loading control.

in PAP I⁻ mutant (>5-fold; Figure 9B). This large stabilization indicates that polyadenylation is required for the decay of this sRNA. Interestingly, 3′ RACE experiments performed in *E. coli* revealed the existence of 3′ A-tails of different lengths in the SraL transcript (38). The decay of SraL was also slower in the *rne-537* and PNPase⁻ mutants, with a higher stabilization in the *rne-537* (Figure 9A). The wild-type strain and both mutants showed an accumulation of a smear of slightly larger transcripts. This size heterogeneity was absent in Δ*pcnB* mutant (Figure 9B). Previous northern blot analysis of SraL in *E. coli* also showed this effect in a PAP I⁻ mutant (38). This data suggests that the presence of poly(A) tails of different lengths in SraL transcript causes these discrete differences in size. The upper band (band X in Figure 9A) shown to accumulate in PNPase and degradosome mutants was reduced in the wild-type strain. In PAP I⁻ mutant, the primary SraL transcript corresponded to the smaller band (Y), which is a defined sharp band (Figure 9B). We predict that this is due to the absence of transcript polyadenylation in the PAP I⁻ mutant. One striking difference in the RNase III- mutant is that the transcript appeared as a single defined band. The size of this band corresponds to the larger band, X. The levels of SraL were higher in this mutant. In spite of this, the absence of the endoribonuclease (Figure 9B) did not significantly change RNA stability. Alterations in transcription levels should account for those differences in steady-state levels that cannot be explained by stability, since the amount of RNA in a cell is determined by the balance of its transcription and degradation.

Analysis of the Hfq influence on the decay of those small RNAs

In order to determine the influence of Hfq on the stability of these four sRNAs in *Salmonella*, we have analysed its decay in an *hfq* mutant strain. As shown in Figure S2 (Supplementary Data), the absence of Hfq did not seem to significantly affect the decay of CsrB and CsrC. In turn, Hfq mutation strongly destabilized MicA sRNA (6-fold decrease in half-life). Similarly, loss of Hfq function decreased the half-life of SraL ~3-fold.

DISCUSSION

Small RNA function has been studied in *E. coli* K12, and comparatively little is known about these regulators in

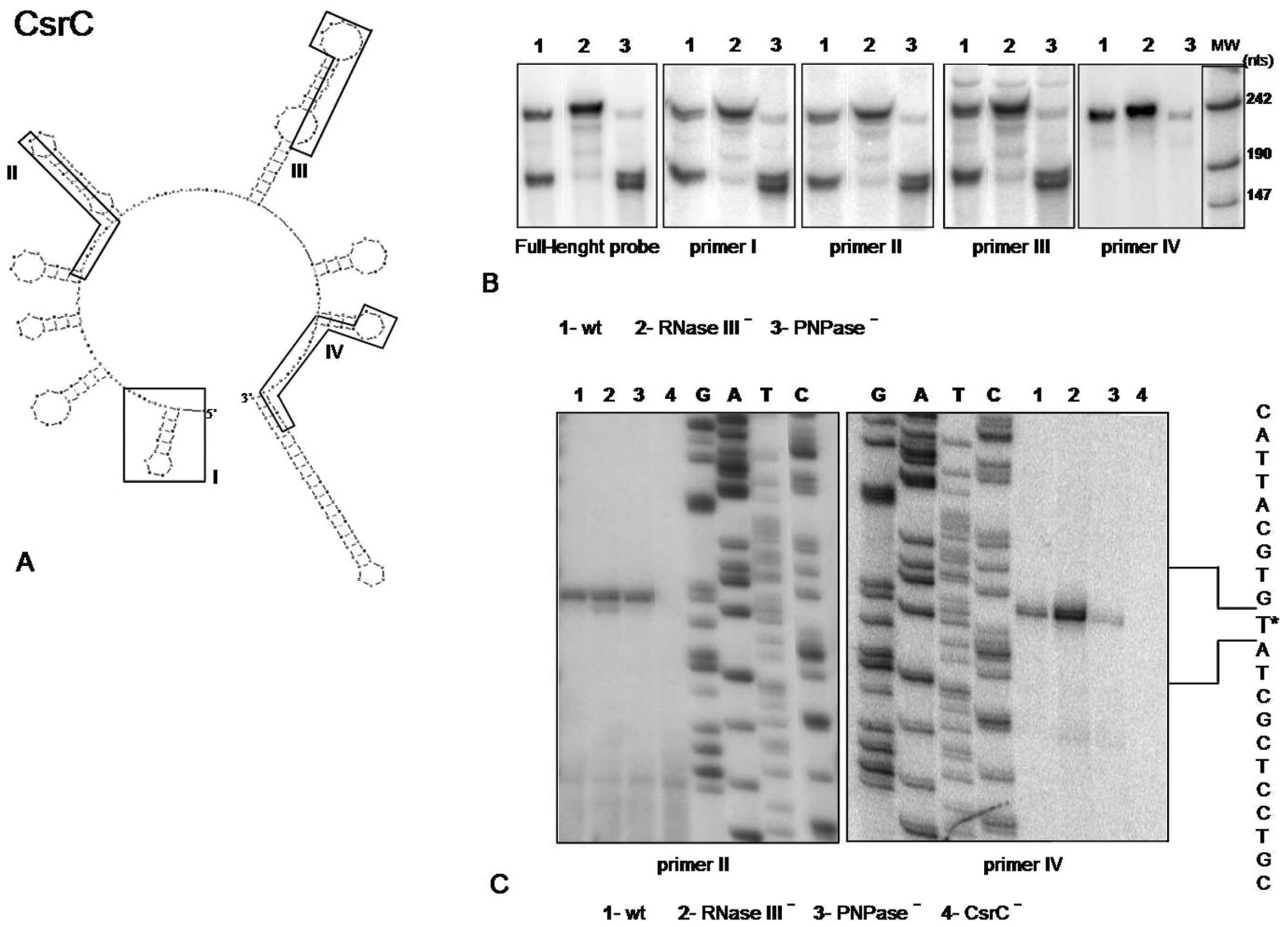


Figure 7. Mapping of CsrC degradation intermediates. (**A**) CsrC sRNA structure representing the approximated location of the different probes used for northern blot and primer extension. Here, I to IV indicate primers CsrC-I to CsrC-IV, respectively. CsrC secondary structure was generated using RNADraw 1.01 based on Ref. (33). (**B**) Northern blot analysis of CsrC RNA in wt, PNPase$^-$ and RNase III$^-$ mutants, with the different probes represented in A. The analysis was done at late stationary-phase OD2 + 6 h. (1) wild-type SL1344, (2) RNase III$^-$ and (3) PNPase$^-$. (**C**) Primer extension analysis using the radiolabelled primers CsrC-II and CsrC-IV, that were annealed to total RNA from SL1344 (wt) and isogenic PNPase$^-$ and RNase III$^-$ mutants. The reaction product of this analysis was unique and similar for the two primers used. The asterisk (*) marks the 3′-terminus of the extension product that is coincident with the sequence published by Ref. (33). (1) Wild-type SL1344, (2) RNase III$^-$, (3) PNPase$^-$ and (4) CsrC$^-$.

other enterobacterial species. The analysis of sRNA levels in different growth conditions has revealed that the four sRNAs are highly expressed in late stationary-phase. Moreover, we have obtained valuable information about particular conditions of expression of these sRNAs in *Salmonella*, probably related to its function and targets in this bacterium. Namely, growth in SPI-1 and SPI-2 inducing media induced the expression of CsrB, CsrC, MicA and SraL. The induction of these sRNAs under those conditions may indicate a relation with virulence functions. It is worthwhile mentioning that considerable differences in the expression of sRNAs have been reported in *E. coli* and *Salmonella*, probably related to their specific role in each bacterium (63,64).

The *Salmonella* CsrB and CsrC sRNAs share strong sequence homology with their respective *E. coli* counterparts, and have been shown to act as CsrA antagonists. In *E. coli*, CsrA is foremost known as a global regulator of carbon metabolism (65,66). In *E. coli*, CsrA is a global regulator of carbon metabolism. In *Salmonella*, it has been shown to regulate specialized virulence determinants not found in *E. coli* (32,67). The CsrB and CsrC expression patterns reported here are in good agreement with the proposed function of these sRNAs as antagonists of CsrA. This protein negatively controls the SPI-1 encoded virulence genes that allow *Salmonella* to invade non-phagocytic cells. CsrB and CsrC are upregulated in SPI-1 media as well as in early stationary phase (OD_{600} of 2), the other condition known to induce the invasion genes. They may therefore act to alleviate the CsrA repression of invasion genes and ensure an optimal epithelial invasion by *Salmonella* (63). In contrast, both sRNAs are repressed in SPI-2 media, a condition that negatively regulates invasion genes and induces the SPI-2 virulence factors needed for intra-macrophage survival and systemic disease.

SraL sRNA was originally identified in *E. coli*, and in this report we show that it is also expressed in *Salmonella*.

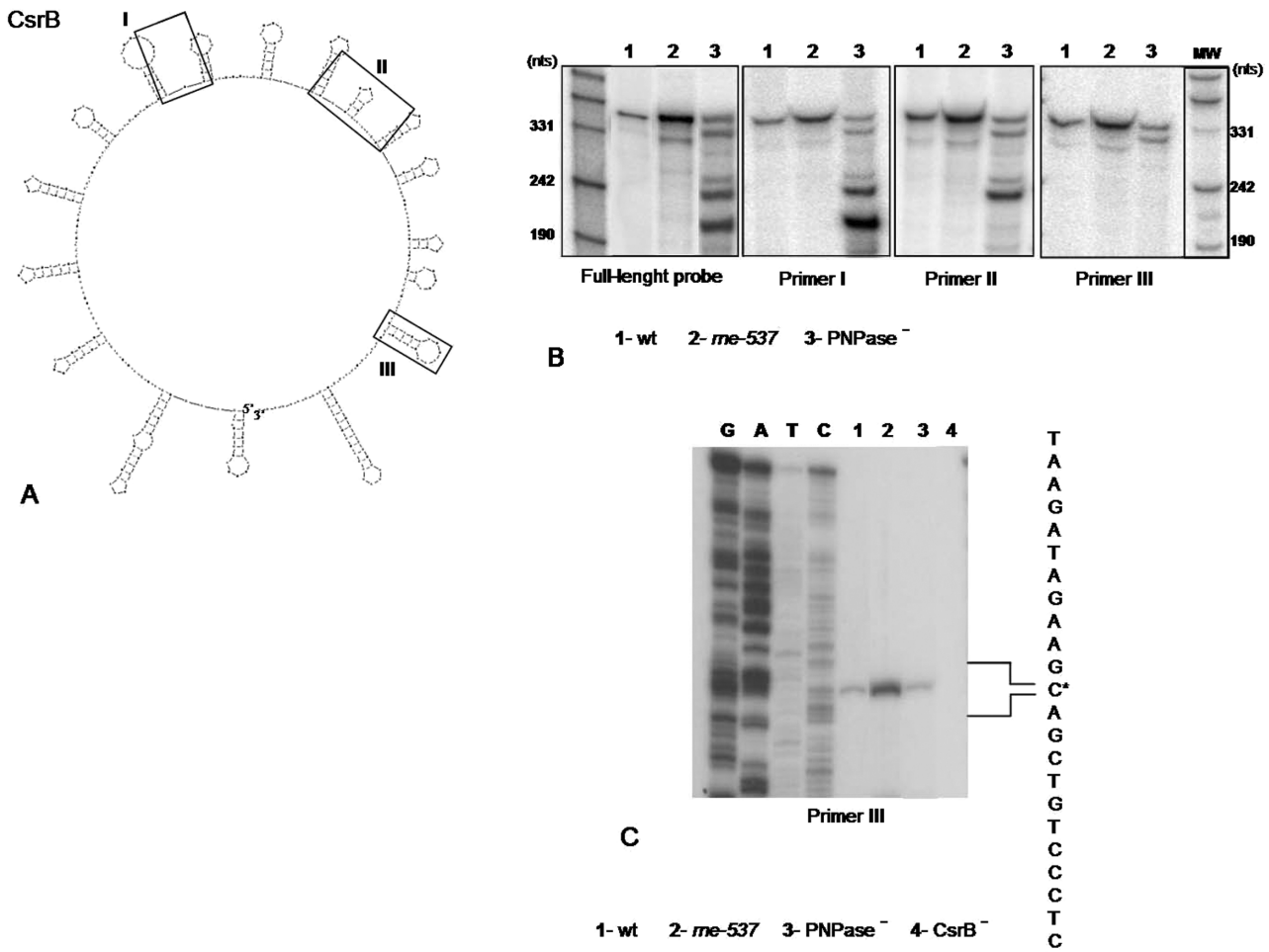


Figure 8. Mapping of CsrB degradation intermediates. (**A**) CsrB sRNA structure representing the approximated location of the different probes used for northern blot and primer extension. A to C indicate primers CsrB-I to CsrB-III, respectively. CsrC secondary structure was generated using RNADraw 1.01 based on Ref. (32). (**B**) Northern blot analysis of CsrB RNA in wt, PNPase and *rne-537* mutants, with the different probes represented in A. The analysis was done at late stationary-phase OD2 + 6 h. (1) wild-type SL1344, (2) *rne-537* and (3) PNPase$^-$. (**C**) Primer extension analysis using the radiolabelled primer CsrB$^-$III that was annealed to total RNA from SL1344 (wt) and isogenic PNPase$^-$ and *rne-537* mutants. The reaction product of this analysis was unique and similar for the two primers used. The asterisk (*) marks the 3′-terminus of the extension product that is coincident with the sequence published by Ref. (32). (1) Wild-type SL1344, (2) *rne-537*, (3) PNPase$^-$ and (4) CsrB$^-$.

The factors that drive *sraL* transcription are unknown yet. We have observed that SraL levels are highly accumulated in stationary phase and SPI-2 inducing conditions. The accumulation under SPI-2 induction indicates a possible role for this sRNA in *Salmonella* virulence, in particular, after internalization of *Salmonella* into host cells.

Interestingly, the levels of the stationary phase-specific MicA sRNA were also high in SPI-2 induction conditions. Up-regulation of the σ^E regulon, which facilitates the envelope stress response, was previously reported upon macrophage infection (68), the condition that SPI-2 medium is meant to mimic. It is well established that *micA* expression is strictly dependent on the alternative sigma factor, σ^E (61,62,69,70). The raise of MicA levels in SPI-2 medium may be a consequence of the induction of σ^E under this condition.

Our analysis of sRNA processing and decay showed that the degradosome is required for the decay of the sRNAs studied here. That is, an *rne* mutation impairing degradosome formation strongly increased the half-life of the four sRNAs. Nevertheless, we observed that other factors contribute differently to sRNA decay. We propose that RNase E and PNPase cooperate in the decay of these two sRNAs via the degradosome. In this model, CsrB and CsrC decay is most probably initiated by RNase E, since the mutation in the C-terminal scaffold of the enzyme caused a strong stabilization of the transcripts. Moreover, the other endoribonucleases analysed (G and III) had no significant effect on CsrB and CsrC decay. Both sRNAs are highly structured molecules; in *Salmonella* CsrB has 16 predicted stem-loops (32) and CsrC has 8 (33). Some of these stem-loops carry the AGGA motif, similar to an RBS, the putative recognition site for CsrA on its target messages. Several characterized sRNAs have in its sequence a *rho*-independent terminator (71). Both CsrB and CsrC have a 3′-terminal stem-loop characteristic of *rho*-independent terminators. During CsrB and CsrC decay several endonucleolytical cleavages must occur,

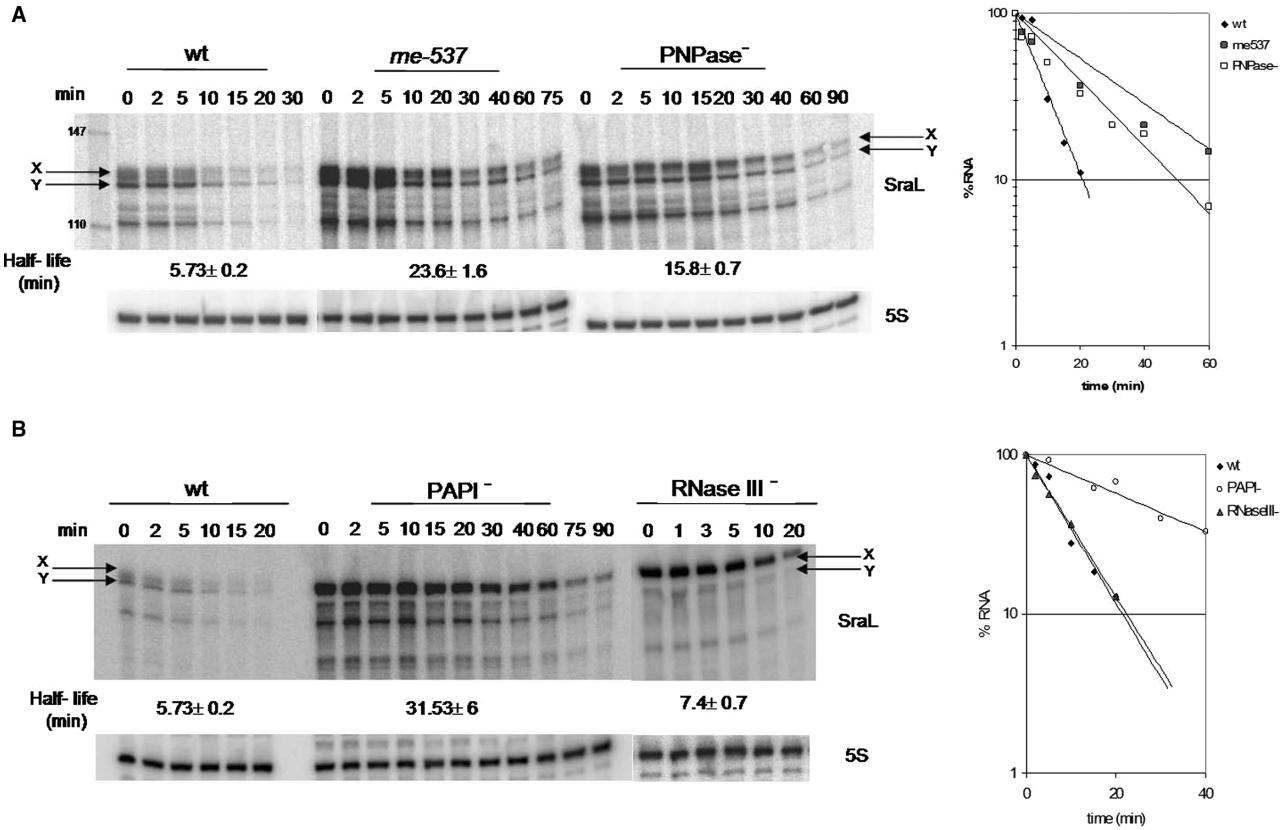


Figure 9. The role of RNase E, PNPase, RNase III and PAPI in SraL regulation. (**A**) Comparison of *rne-537* and PNPase mutations in SraL decay. (**B**) Analysis of the effect of RNase III and PAP I in the decay and processing of SraL transcript.

followed by exonucleolytical cleavage by PNPase. PNPase was shown to be a key factor in the decay of the CsrB and CsrC sRNAs in *Salmonella*, similar to a recent observation in *E. coli* (72). The absence of this exoribonuclease caused a considerable change of the CsrB and CsrC degradation patterns with the concomitant accumulation of several decay intermediates. Primer extension and northern blot analysis of CsrB and CsrC sRNAs showed that the accumulating intermediates have different 3′ ends. RNA degradation pathways typically require endoribonucleolytic cleavages followed by the action of non-specific 3′–5′ processive exoribonucleases. Exoribonucleases can have different specificities over substrates and in some cases there is the accumulation of stable intermediates in the absence of a single exoribonuclease (23,73,74). Purified PNPase is unable to digest through extensive secondary structures (75). However, *in vivo* association of PNPase with an RNA helicase can contribute to PNPase degradation through highly structured RNAs. Moreover, it has been proposed that PAP I facilitates the degradation of highly folded intermediates by providing a 3′ toehold for the progression of the enzyme (76). However, we have seen that the loss of PAP I activity did not affect the stability of either of these two sRNAs (Figure S3 in Supplementary Data) indicating that in this case, polyadenylation of these transcripts is not necessary for exonucleolytic activity. We have mentioned earlier that RNase III did not have an effect on CsrB and CsrC stability. However, in the case of CsrC, the processing of the sRNA is RNase III dependent. In the wild-type strain, two bands are visible for this sRNA. The second band is RNase III dependent and accumulates in PNPase$^-$ mutant. The 5′ end analysis of CsrC in both strains revealed similar 5′ ends. Therefore RNase III must initially process CsrC at one of the 3′ longer stems, generating this second band. It is not known at what level this fragment is necessary for sRNA activity.

We have also analysed if CsrB and CsrC stability depends on Hfq. Analysis of their decay in an *hfq* mutant revealed that Hfq is not needed for the stability of these two sRNAs. This is in agreement with *E. coli* data for these two sRNAs (39,58,72). Since CsrB and CsrC belong to the class of protein regulator sRNAs, a dependence on Hfq was not expected.

We have also studied in detail the decay of SraL in our mutant strains and have found that in the absence of PNPase activity and degradosome assembly, there was a slower decay of the sRNA with a concomitant accumulation of a smear of slightly larger transcripts (most likely polyadenylated precursors). In the PAP I$^-$ mutant, SraL is remarkably stabilized and the bands corresponding to longer SraL molecules were absent, supporting that SraL is polyadenylated. The absence of RNase III caused the accumulation of a larger band of defined length. Several internal cleavage sites were previously mapped in *E. coli* SraL (38). RNase III could be the enzyme responsible for

the initial cut in SraL, possibly within the SraL terminator, which overlaps the terminator of *soxR* encoded on the opposite strand. After RNase III cleavage, RNase E and PNPase may act cooperatively in the transcript decay with the help of PAP I polymerase. It is known that poly(A) tails are the preferred substrate for PNPase and accelerate the decay process. A similar mechanism of decay was previously reported for the degradation of the plasmid-encoded RNAI (28,77) and for RNAs that regulate replication and partition of R1 plasmids (78,79).

According to our data, Hfq stabilizes SraL ~3-fold. Wassarman and co-workers (58) were not able to confirm Hfq binding to SraL in their Hfq co-immunoprecipitation analysis in *E. coli*. Whilst SraL mechanism and targets have yet to be revealed, our results indicate that SraL belongs to the group of Hfq-dependent sRNAs.

MicA turnover was seen to be significantly dependent on degradosome and PNPase. *ompA* mRNA is the main MicA target. The rate-limiting step in the decay of this message was assigned to endoribonuclease E (80). PNPase was also shown to be one of the exoribonucleases affecting *ompA* mRNA in stationary-phase (37). This suggests that the same enzymes are responsible for the regulation of the sRNA and the respective target. However, other targets are being discovered for this sRNA. A very recent report shows that MicA downregulates expression of *lamB* gene in *Salmonella*, also in a Hfq-dependent way (36). Additionally, MicA may also interact with the 5′ UTR of *luxS* to which it is transcribed in opposite direction (81). Interestingly, we have seen that in the absence of RNase III MicA is extremely stable. RNase III can recognize and cleave perfect RNA duplexes formed by interacting RNAs. The regulation of MicA by RNase III may involve the interaction with its target RNA, since MicA forms an extended RNA duplex that is close to the length ideal for RNase III substrates. This could implicate the coupling of sRNA-target regulation, as previously reported for RyhB sRNA (6). However, the unaltered stability of MicA in the absence of *ompA* (Figure S4 in Supplementary Data) shows that MicA degradation is independent of *omp*A. Regarding Hfq influence on the turnover of the four sRNAs analysed, MicA showed the strongest dependence on Hfq for stability. It is known that the MicA-dependent decay of *omp*A-mRNA depends on Hfq. *In vitro* studies revealed that Hfq facilitates binding of the regulatory RNA to the translational initiation region of this target (35). Our results indicate that Hfq is also involved in protecting MicA from degradation in *Salmonella*.

Few reports have shown an involvement of endoribonuclease III in bacterial sRNA decay. However, it is known that enzymes of the RNase III family are key players in the mechanisms of regulation of noncoding RNAs in eukaryotes (82). These enzymes, specific for double-stranded RNAs, are essential in the biogenesis of the eukaryotic noncoding RNAs that participate in the process of RNA Interference (miRNAs, siRNAs). A role for this enzyme was also expected in bacterial sRNA regulation. In fact, it was reported that RNase III is responsible for the cleavage of *tisAB* mRNA upon IstR-1 sRNA binding, in *E. coli* (54,83). In addition, it was proposed (84) that RyhB sRNA decay *in vivo* is dependent on this endoribonuclease upon base pairing of the sRNA to the 5′-UTR of its mRNA target. Similarly, RNase III also contributes to the negative control of *spa* (encoding the surface protein A) and other virulence factor-encoding mRNAs by the regulatory RNAIII in the Gram-positive pathogen, *Staphylococcus aureus* (85,86). Our results in *Salmonella* show that the effect of RNase III varied among the sRNAs studied.

The *Salmonella* mutants deficient in enzymes that affect sRNA and mRNA turnover will be very important for post-transcriptional studies in this bacterial model pathogen. The work presented here has identified some of the enzymes directly involved in the decay of sRNAs. We conclude that sRNA decay cannot be easily generalized. The role of each of the enzymes cooperating in sRNA turnover depends on the specific sRNA and its respective decay mechanism.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

REFERENCES

1. Majdalani,N., Vanderpool,C.K. and Gottesman,S. (2005) Bacterial small RNA regulators. *Crit. Rev. Biochem. Mol. Biol.*, **40**, 93–113.
2. Storz,G., Altuvia,S. and Wassarman,K.M. (2005) An abundance of RNA regulators. *Annu. Rev. Biochem.*, **74**, 199–217.
3. Vogel,J. and Sharma,C.M. (2005) How to find small non-coding RNAs in bacteria. *Biol. Chem.*, **386**, 1219–1238.
4. Hershberg,R., Altuvia,S. and Margalit,H. (2003) A survey of small RNA-encoding genes in *Escherichia coli*. *Nucleic Acids Res.*, **31**, 1813–1820.
5. Storz,G., Opdyke,J.A. and Zhang,A. (2004) Controlling mRNA stability and translation with small, noncoding RNAs. *Curr. Opin. Microbiol.*, **7**, 140–144.
6. Massé,E., Escorcia,F.E. and Gottesman,S. (2003) Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. *Genes Dev.*, **17**, 2374–2383.
7. Rasmussen,A.A., Eriksen,M., Gilany,K., Udesen,C., Franch,T., Petersen,C. and Valentin-Hansen,P. (2005) Regulation of ompA mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. *Mol. Microbiol.*, **58**, 1421–1429.

8. Valentin-Hansen,P., Eriksen,M. and Udesen,C. (2004) The bacterial Sm-like protein Hfq: a key player in RNA transactions. *Mol. Microbiol.*, **51**, 1525–1533.
9. Aiba,H. (2007) Mechanism of RNA silencing by Hfq-binding small RNAs. *Curr. Opin. Microbiol.*, **10**, 134–139.
10. Urban,J.H. and Vogel,J. (2007) Translational control and target recognition by *Escherichia coli* small RNAs in vivo. *Nucleic Acids Res.*, **35**, 1018–1037.
11. Vogel,J., Bartels,V., Tang,T.H., Churakov,G., Slagter-Jager,J.G., Huttenhofer,A. and Wagner,E.G. (2003) RNomics in *Escherichia coli* detects new sRNA species and indicates parallel transcriptional output in bacteria. *Nucleic Acids Res.*, **31**, 6435–6443.
12. Régnier,P. and Arraiano,C.M. (2000) Degradation of mRNA in bacteria: emergence of ubiquitous features. *Bioessays*, **22**, 235–244.
13. Arraiano,C.M. and Maquat,L.E. (2003) Post-transcriptional control of gene expression: effectors of mRNA decay. *Mol. Microbiol.*, **49**, 267–276.
14. Carpousis,A.J., Van Houwe,G., Ehretsmann,C. and Krisch,H.M. (1994) Copurification of *E. coli* RNAase E and PNPase: evidence for a specific association between two enzymes important in RNA processing and degradation. *Cell*, **76**, 889–900.
15. Carpousis,A.J., Vanzo,N.F. and Raynal,L.C. (1999) mRNA degradation. A tale of poly(A) and multiprotein machines. *Trends Genet.*, **15**, 24–28.
16. Lee,K., Bernstein,J.A. and Cohen,S.N. (2002) RNase G complementation of *rne* null mutation identifies functional interrelationships with RNase E in *Escherichia coli*. *Mol. Microbiol.*, **43**, 1445–1456.
17. Li,Z., Pandit,S. and Deutscher,M.P. (1999) RNase G (CafA protein) and RNase E are both required for the 5′ maturation of 16S ribosomal RNA. *EMBO J.*, **18**, 2878–2885.
18. Ow,M.C., Perwez,T. and Kushner,S.R. (2003) RNase G of *Escherichia coli* exhibits only limited functional overlap with its essential homologue, RNase E. *Mol. Microbiol.*, **49**, 607–622.
19. Nicholson,A.W. (1999) Function, mechanism and regulation of bacterial ribonucleases. *FEMS Microbiol. Rev.*, **23**, 371–390.
20. Santos,J.M., Drider,D., Marujo,P.E., Lopez,P. and Arraiano,C.M. (1997) Determinant role of *E. coli* RNase III in the decay of both specific and heterologous mRNAs. *FEMS Microbiol. Lett.*, **157**, 31–38.
21. Régnier,P. and Grunberg-Manago,M. (1990) RNase III cleavages in non-coding leaders of *Escherichia coli* transcripts control mRNA stability and genetic expression. *Biochimie*, **72**, 825–834.
22. Frazão,C., McVey,C.E., Amblar,M., Barbas,A., Vonrhein,C., Arraiano,C.M. and Carrondo,M.A. (2006) Unravelling the dynamics of RNA degradation by ribonuclease II and its RNA-bound complex. *Nature*, **443**, 110–114.
23. Amblar,M., Barbas,A., Gomez-Puertas,P. and Arraiano,C.M. (2007) The role of the S1 domain in exoribonucleolytic activity: substrate specificity and multimerization. *RNA*, **13**, 317–327.
24. Mohanty,B.K. and Kushner,S.R. (2000) Polynucleotide phosphorylase functions both as a 3′ right-arrow 5′ exonuclease and a poly(A) polymerase in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **97**, 11966–11971.
25. Cairrão,F., Cruz,A., Mori,H. and Arraiano,C.M. (2003) Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. *Mol. Microbiol.*, **50**, 1349–1360.
26. Sarkar,N. (1997) Polyadenylation of mRNA in prokaryotes. *Annu. Rev. Biochem.*, **66**, 173–197.
27. Hajnsdorf,E., Braun,F., Haugel-Nielsen,J. and Regnier,P. (1995) Polyadenylylation destabilizes the *rps*O mRNA of *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **92**, 3973–3977.
28. Xu,F. and Cohen,S.N. (1995) RNA degradation in *Escherichia coli* regulated by 3′ adenylation and 5′ phosphorylation. *Nature*, **374**, 180–183.
29. O'Hara,E.B., Chekanova,J.A., Ingle,C.A., Kushner,Z.R., Peters,E. and Kushner,S.R. (1995) Polyadenylylation helps regulate mRNA decay in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **92**, 1807–1811.
30. Marujo,P.E., Hajnsdorf,E., Le Derout,J., Andrade,R., Arraiano,C.M. and Régnier,P. (2000) RNase II removes the oligo(A) tails that destabilize the *rps*O mRNA of *Escherichia coli*. *RNA*, **6**, 1185–1193.
31. Santos,J.M., Freire,P., Mesquita,F.S., Mika,F., Hengge,R. and Arraiano,C.M. (2006) Poly(A)-polymerase I links transcription with mRNA degradation via sigmaS proteolysis. *Mol. Microbiol.*, **60**, 177–188.
32. Altier,C., Suyemoto,M., Ruiz,A.I., Burnham,K.D. and Maurer,R. (2000) Characterization of two novel regulatory genes affecting *Salmonella* invasion gene expression. *Mol. Microbiol.*, **35**, 635–646.
33. Fortune,D.R., Suyemoto,M. and Altier,C. (2006) Identification of CsrC and characterization of its role in epithelial cell invasion in *Salmonella enterica* serovar Typhimurium. *Infect. Immun.*, **74**, 331–339.
34. Liu,M.Y., Gui,G., Wei,B., Preston,J.F.III, Oakford,L., Yuksel,U., Giedroc,D.P. and Romeo,T. (1997) The RNA molecule CsrB binds to the global regulatory protein CsrA and antagonizes its activity in *Escherichia coli*. *J. Biol. Chem.*, **272**, 17502–17510.
35. Udekwu,K.I., Darfeuille,F., Vogel,J., Reimegard,J., Holmqvist,E. and Wagner,E.G. (2005) Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. *Genes Dev.*, **19**, 2355–2366.
36. Bossi,L. and Figueroa-Bossi,N. (2007) A small RNA downregulates LamB maltoporin in *Salmonella*. *Mol. Microbiol.*, **65**, 799–810.
37. Andrade,J.M., Cairrão,F. and Arraiano,C.M. (2006) RNase R affects gene expression in stationary phase: regulation of ompA. *Mol. Microbiol.*, **60**, 219–228.
38. Argaman,L., Hershberg,R., Vogel,J., Bejerano,G., Wagner,E.G., Margalit,H. and Altuvia,S. (2001) Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli*. *Curr. Biol.*, **11**, 941–950.
39. Zhang,A., Wassarman,K.M., Rosenow,C., Tjaden,B.C., Storz,G. and Gottesman,S. (2003) Global analysis of small RNA and mRNA targets of Hfq. *Mol. Microbiol.*, **50**, 1111–1124.
40. Datsenko,K.A. and Wanner,B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl Acad. Sci. USA*, **97**, 6640–6645.
41. Schmieger,H. (1971) The fate of the bacterial chromosome in P22-infected cells of *Salmonella typhimurium*. *Mol. Gen. Genet.*, **110**, 238–244.
42. Sittka,A., Pfeiffer,V., Tedin,K. and Vogel,J. (2007) The RNA chaperone Hfq is essential for the virulence of *Salmonella typhimurium*. *Mol. Microbiol.*, **63**, 193–217.
43. Lober,S., Jackel,D., Kaiser,N. and Hensel,M. (2006) Regulation of *Salmonella* pathogenicity island 2 genes by independent environmental signals. *Int. J. Med. Microbiol.*, **296**, 435–447.
44. McClelland,M., Sanderson,K.E., Spieth,J., Clifton,S.W., Latreille,P., Courtney,L., Porwollik,S., Ali,J., Dante,M. *et al.* (2001) Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature*, **413**, 852–856.
45. Arraiano,C.M., Yancey,S.D. and Kushner,S.R. (1988) Stabilization of discrete mRNA breakdown products in *ams pnp rnb* multiple mutants of *Escherichia coli* K-12. *J. Bacteriol.*, **170**, 4625–4633.
46. Viegas,S.C., Schmidt,D., Kasche,V., Arraiano,C.M. and Ignatova,Z. (2005) Effect of the increased stability of the penicillin amidase mRNA on the protein expression levels. *FEBS Lett.*, **579**, 5069–5073.
47. Cairrão,F. and Arraiano,C.M. (2006) The role of endoribonucleases in the regulation of RNase R. *Biochem. Biophys. Res. Commun.*, **343**, 731–737.
48. Vanzo,N.F., Li,Y.S., Py,B., Blum,E., Higgins,C.F., Raynal,L.C., Krisch,H.M. and Carpousis,A.J. (1998) Ribonuclease E organizes the protein interactions in the Escherichia coli RNA degradosome. *Genes Dev.*, **12**, 2770–2781.
49. Lopez,P.J., Marchand,I., Joyce,S.A. and Dreyfus,M. (1999) The C-terminal half of RNase E, which organizes the *Escherichia coli* degradosome, participates in mRNA degradation but not rRNA processing in vivo. *Mol. Microbiol.*, **33**, 188–199.
50. Ow,M.C., Liu,Q. and Kushner,S.R. (2000) Analysis of mRNA decay and rRNA processing in *Escherichia coli* in the absence of RNase E-based degradosome assembly. *Mol. Microbiol.*, **38**, 854–866.
51. Amblar,M., Viegas,S.C., López,P. and Arraiano,C.M. (2004) Homologous and heterologous expression of RNase III

from *Lactococcus lactis*. *Biochem. Biophys. Res. Commun.*, **323**, 884–890.
52. Burgin,A.B., Parodos,K., Lane,D.J. and Pace,N.R. (1990) The excision of intervening sequences from *Salmonella* 23S ribosomal RNA. *Cell*, **60**, 405–414.
53. Altuvia,S., Weinstein-Fischer,D., Zhang,A., Postow,L. and Storz,G. (1997) A small, stable RNA induced by oxidative stress: role as a pleiotropic regulator and antimutator. *Cell*, **90**, 43–53.
54. Vogel,J., Argaman,L., Wagner,E.G. and Altuvia,S. (2004) The small RNA IstR inhibits synthesis of an SOS-induced toxic peptide. *Curr. Biol.*, **14**, 2271–2276.
55. Sledjeski,D.D., Gupta,A. and Gottesman,S. (1996) The small RNA, DsrA, is essential for the low temperature expression of RpoS during exponential growth in *Escherichia coli*. *EMBO J.*, **15**, 3993–4000.
56. Massé,E. and Gottesman,S. (2002) A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **99**, 4620–4625.
57. Delihas,N. and Forst,S. (2001) MicF: an antisense RNA gene involved in response of *Escherichia coli* to global stress factors. *J. Mol. Biol.*, **313**, 1–12.
58. Wassarman,K.M., Repoila,F., Rosenow,C., Storz,G. and Gottesman,S. (2001) Identification of novel small RNAs using comparative genomics and microarrays. *Genes Dev.*, **15**, 1637–1651.
59. Song,M., Kim,H.J., Kim,E.Y., Shin,M., Lee,H.C., Hong,Y., Rhee,J.H., Yoon,H., Ryu,S. *et al.* (2004) ppGpp-dependent stationary phase induction of genes on *Salmonella* pathogenicity island 1. *J. Biol. Chem.*, **279**, 34183–34190.
60. Lee,C.A. and Falkow,S. (1990) The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. *Proc. Natl Acad. Sci. USA*, **87**, 4304–4308.
61. Figueroa-Bossi,N., Lemire,S., Maloriol,D., Balbontin,R., Casadesus,J. and Bossi,L. (2006) Loss of Hfq activates the sigmaE-dependent envelope stress response in *Salmonella enterica*. *Mol. Microbiol.*, **62**, 838–852.
62. Papenfort,K., Pfeiffer,V., Mika,F., Lucchini,S., Hinton,J.C. and Vogel,J. (2006) SigmaE-dependent small RNAs of *Salmonella* respond to membrane stress by accelerating global omp mRNA decay. *Mol. Microbiol.*, **62**, 1674–1688.
63. Altier,C., Suyemoto,M. and Lawhon,S.D. (2000) Regulation of *Salmonella enterica* serovar typhimurium invasion genes by csrA. *Infect. Immun.*, **68**, 6790–6797.
64. Jones,A.M., Goodwill,A. and Elliott,T. (2006) Limited role for the DsrA and RprA regulatory RNAs in *rpo*S regulation in *Salmonella enterica*. *J. Bacteriol.*, **188**, 5077–5088.
65. Romeo,T., Gong,M., Liu,M.Y. and Brun-Zinkernagel,A.M. (1993) Identification and molecular characterization of csrA, a pleiotropic gene from Escherichia coli that affects glycogen biosynthesis, gluconeogenesis, cell size, and surface properties. *J. Bacteriol.*, **175**, 4744–4755.
66. Liu,M.Y., Yang,H. and Romeo,T. (1995) The product of the pleiotropic Escherichia coli gene csrA modulates glycogen biosynthesis via effects on mRNA stability. *J. Bacteriol.*, **177**, 2663–2672.
67. Lawhon,S.D., Frye,J.G., Suyemoto,M., Porwollik,S., McClelland,M. and Altier,C. (2003) Global regulation by CsrA in *Salmonella typhimurium*. *Mol. Microbiol.*, **48**, 1633–1645.
68. Eriksson,S., Lucchini,S., Thompson,A., Rhen,M. and Hinton,J.C. (2003) Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. *Mol. Microbiol.*, **47**, 103–118.
69. Johansen,J., Rasmussen,A.A., Overgaard,M. and Valentin-Hansen,P. (2006) Conserved small non-coding RNAs that belong to the sigmaE regulon: role in down-regulation of outer membrane proteins. *J. Mol. Biol.*, **364**, 1–8.
70. Udekwu,K.I. and Wagner,E.G. (2007) Sigma E controls biogenesis of the antisense RNA MicA. *Nucleic Acids Res.*, **35**, 1279–1288.
71. Davis,B.M. and Waldor,M.K. (2007) RNase E-dependent processing stabilizes MicX, a *Vibrio cholerae* sRNA. *Mol. Microbiol.*, **65**, 373–385.
72. Suzuki,K., Babitzke,P., Kushner,S.R. and Romeo,T. (2006) Identification of a novel regulatory protein (CsrD) that targets the global regulatory RNAs CsrB and CsrC for degradation by RNase E. *Genes Dev.*, **20**, 2605–2617.
73. Cheng,Z.F. and Deutscher,M.P. (2005) An important role for RNase R in mRNA decay. *Mol. Cell*, **17**, 313–318.
74. Mackie,G.A. (1998) Ribonuclease E is a 5′-end-dependent endonuclease. *Nature*, **395**, 720–723.
75. Vincent,H.A. and Deutscher,M.P. (2006) Substrate recognition and catalysis by the exoribonuclease RNase R. *J. Biol. Chem.*, **281**, 29769–29775.
76. Coburn,G.A. and Mackie,G.A. (1998) Reconstitution of the degradation of the mRNA for ribosomal protein S20 with purified enzymes. *J. Mol. Biol.*, **279**, 1061–1074.
77. Cohen,S.N. (1995) Surprises at the 3′ end of prokaryotic RNA. *Cell*, **80**, 829–832.
78. Dam Mikkelsen,N. and Gerdes,K. (1997) Sok antisense RNA from plasmid R1 is functionally inactivated by RNase E and poly-adenylated by poly(A) polymerase I. *Mol. Microbiol.*, **26**, 311–320.
79. Soderbom,F., Binnie,U., Masters,M. and Wagner,E.G. (1997) Regulation of plasmid R1 replication: PcnB and RNase E expedite the decay of the antisense RNA,CopA. *Mol. Microbiol.*, **26**, 493–504.
80. Melefors,O. and von Gabain,A. (1988) Site-specific endonucleolytic cleavages and the regulation of stability of *E. coli* ompA mRNA. *Cell*, **52**, 893–901.
81. Udekwu,K.I. (2007) Ph.D. thesis, Uppsala University, Uppsala.
82. Carmell,M.A. and Hannon,G.J. (2004) RNase III enzymes and the initiation of gene silencing. *Nat. Struct. Mol. Biol.*, **11**, 214–218.
83. Darfeuille,F., Unoson,C., Vogel,J. and Wagner,E.G. (2007) An antisense RNA inhibits translation by competing with standby ribosomes. *Mol. Cell*, **26**, 381–392.
84. Afonyushkin,T., Vecerek,B., Moll,I., Blasi,U. and Kaberdin,V.R. (2005) Both RNase E and RNase III control the stability of sodB mRNA upon translational inhibition by the small regulatory RNA RyhB. *Nucleic Acids Res.*, **33**, 1678–1689.
85. Huntzinger,E., Boisset,S., Saveanu,C., Benito,Y., Geissmann,T., Namane,A., Lina,G., Etienne,J., Ehresmann,B. *et al.* (2005) *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression. *EMBO J.*, **24**, 824–835.
86. Boisset,S., Geissmann,T., Huntzinger,E., Fechter,P., Bendridi,N., Possedko,M., Chevalier,C., Helfer,A.C., Benito,Y. *et al.* (2007) *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. *Genes Dev.*, **21**, 1353–1366.
87. Hoiseth,S.K. and Stocker,B.A. (1981) Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature*, **291**, 238–239.

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Regulation of the small regulatory RNA MicA by ribonuclease III: a target-dependent pathway

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ABSTRACT

MicA is a *trans*-encoded small non-coding RNA, which downregulates porin-expression in stationary-phase. In this work, we focus on the role of endoribonucleases III and E on *Salmonella typhimurium* sRNA MicA regulation. RNase III is shown to regulate MicA in a target-coupled way, while RNase E is responsible for the control of free MicA levels in the cell. We purified both *Salmonella* enzymes and demonstrated that *in vitro* RNase III is only active over MicA when in complex with its targets (whether *ompA* or *lamB* mRNAs). *In vivo*, MicA is demonstrated to be cleaved by RNase III in a coupled way with *ompA* mRNA. On the other hand, RNase E is able to cleave unpaired MicA and does not show a marked dependence on its 5′ phosphorylation state. The main conclusion of this work is the existence of two independent pathways for MicA turnover. Each pathway involves a distinct endoribonuclease, having a different role in the context of the fine-tuned regulation of porin levels. Cleavage of MicA by RNase III in a target-dependent fashion, with the concomitant decay of the mRNA target, strongly resembles the eukaryotic RNAi system, where RNase III-like enzymes play a pivotal role.

INTRODUCTION

Small non-coding RNAs (sRNAs) play very important roles in post-transcriptional control of gene expression. MicF was the first *trans*-encoded antisense sRNA described and was discovered a little more than a quarter-century ago as a regulator of the *Escherichia coli ompF* mRNA (1). Following the advent of systematic genome wide sRNA searches, the total number of known sRNAs in *E. coli* and the model pathogen *Salmonella enterica* serovar Typhimurium has grown to well over a hundred (2).

An extensive network of *trans*-antisense sRNAs have been shown to downregulate the expression of several outer membrane proteins (OMPs). While in some cases the same sRNA regulates multiple *omp* mRNAs (3,4), in other cases the same *omp* mRNA is target of multiple sRNAs (4–6). OMPs are embedded within the outer membrane, which together with the peptidoglycan layer and the inner membrane form the bacterial cell envelope, the first barrier of defense against external aggressions. Coordination in the expression of *omp* genes seems critical for proper envelope assembly, and accounts for the existence of so many sRNAs to regulate OMP mRNAs.

To survive in a changing environment, bacteria must constantly adjust the nature and abundance of surface components. Any condition that unbalances OMP levels activates the response of the transcription factor σ^E (7,8) that triggers transcription of a set of genes, which collectively help the bacterium to recover from the stress condition. MicA and RybB are two of the σ^E activated genes in stationary phase, whose role is to immediately limit OMP synthesis (4,6,9). Both sRNAs act in the same fashion: they inhibit protein synthesis by base pairing to the translation initiation region of their mRNA targets in an Hfq-dependent manner, followed by the subsequent degradation of the mRNA. Although sRNAs generally modulate translational initiation by interfering with 30S ribosome loading, alterations of target mRNA levels are also often observed (10,11). A few studies performed in *E. coli* suggest that RNase cleavage of target mRNAs may be directly coupled to the degradation of the sRNA that is regulating the process, with both RNAs being degraded upon sRNA action (12–14).

RNases can have a major impact on sRNAs regulatory pathways by performing a key role in the biogenesis and processing of sRNAs, as well as in controlling their cellular levels through regulation of their turnover

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(12,15–19). In *E. coli*, and presumably in many other Gram-negative bacteria, including *Salmonella*, mRNA decay is normally initiated by an endonucleolytic cleavage mainly performed by RNase E (20) and, sometimes by RNase III (21), followed by exoribonucleolytic degradation (19,22). In *E. coli*, both endoribonucleases have also been implicated in the decay of sRNAs, upon translational silencing (23).

We have previously reported specific contributions of several *Salmonella* ribonucleases on the turnover of different sRNAs (17). In this work, we have cloned and purified for the first time *Salmonella* RNase III and RNase E and have demonstrated that both endoribonucleases are responsible for the control of MicA sRNA levels. The role of the double stranded-specific endoribonuclease III over MicA only occurs through a target-dependent pathway, whether *in vitro* or *in vivo*. By contrast, the single stranded-specific endoribonuclease E is able to efficiently degrade free MicA sRNA. A model is proposed to explain the cooperation of both enzymes in the cell in order to achieve the fine-tuned control of the post-transcriptional regulator MicA.

MATERIALS AND METHODS

Oligonucleotides

All oligonucleotides used in this study are listed in the Supplementary Table S1 and were synthesized by STAB Vida, Portugal.

Bacterial strains

All bacterial strains and plasmids used in this study are listed in the Tables 1 and 2, respectively. All *Salmonella* strains used are isogenic derivates of the wild-type *Salmonella enterica* serovar Typhimurium strain SL1344. The OmpA$^-$ (CMA-552), LamB$^-$ (CMA-554) and MicA$^-$ (CMA-555) mutants were constructed using the primer pairs pSV-104/pSV-105, pSV-108/pSV-109 and pSV-146/pSV-147, respectively, and following the λ-red recombinase method (24), with few modifications, as previously described (17). All chromosomal mutations were subsequently transferred to a fresh SL1344 background by P22 HT105/1 int-201 transduction (25). The chloramphenicol-resistance cassette of plasmid pKD3 replaces nucleotides −190 to +1064 of the *ompA* gene, −20 to +1339 of *lamB* and +8 to +78 of *micA*. All gene deletions were verified by colony PCR using the primer pairs pSV-106/pSV-107 for *ompA*, pSV-110/pSV-111 for *lamB* and pSV-148/pSV-149 for *micA*. The *S. typhimurium* RNase III deficient strain (CMA-551) was obtained by P22 transduction from SA5303 strain (26) and is tetracycline resistant. The double mutants were constructed using the same transduction method.

Bacterial growth

All strains were grown in Luria-Bertani (LB) broth at 37°C with agitation throughout this study. SOC medium (Super Optimal Broth with Catabolite Repression medium) was used to recover transformants after heat shock (in the case of *E. coli*) or electroporation (in the case of *Salmonella*), before plating. Growth medium was supplemented with the following antibiotics when appropriate: ampicillin (150 μg/ml), chloramphenicol (25 μg/ml), streptomycin (90 μg/ml) and tetracycline (25 μg/ml).

RNA extraction and northern blot analysis

Overnight cultures were diluted 1/100 in fresh LB medium and grown until 6 h after OD_{600} of 2 (OD2+6). Culture

Table 1. List of strains used in this work

Strain	Relevant markers/Genotype	Source/Reference
S. typhimurium, SL1344	Str^R*hisG rpsL xyl*	(56)
CMA-537	SL1344 *rne-537* (Δ*rne*::Cm^R)	(17)
CMA-551	SL1344 *rnc-14*::ΔTn*10* (Tc^R)	This study
CMA-552	SL1344 *ompA* (Δ*ompA*::Cm^R)	This study
CMA-554	SL1344 *lamB* (Δ*lamB*::Cm^R)	This study
CMA-555	SL1344 *micA* (Δ*micA*::Cm^R)	This study
CMA-556	SL1344 *rnc-14 micA* (*rnc-14*::ΔTn*10*/Δ*micA*::Cm^R)	This study
CMA-557	SL1344 *rnc-14 ompA* (*rnc-14*::ΔTn*10*/Δ*ompA*::Cm^R)	This study
CMA-558	SL1344 *rnc-14 rne-537* (*rnc-14*::ΔTn*10*/Δ*rne*::Cm^R)	This study
E. coli BL21(DE3)	F$^-$ *omp*T *hsd* S$_B$(*rb*$^-$*mb*$^-$) gal dcm (DE3)	(57)
E. coli BL21(DE3)*rec*A *rnc105*	F$^-$ *omp*T *hsd* S$_B$(*rb*$^-$*mb*$^-$) gal dcm (DE3) *rec*A *rnc*105	(27)
E. coli DH5α	*rec*A1 *end*A1 *gyr*A96 thi-*hsd*R17 *sup*E44 *rel*A1 Δ*lac*ZYA-*arg* FU169 f80dLacZDM15	New England Biolabs

Table 2. List of plasmids used in this work

Plasmid	Comments	Origin/Marker	Reference
pKD3	Template for mutants construction; carries chloramphenicol-resistance cassete	oriRγ/Amp^R	(23)
pKD46	Temperature-sensitive λ-red recombinase expression plasmid	oriR101/Amp^R	(23)
pCP20	Temperature-sensitive FLP recombinase expression plasmid	Amp^R, Cm^R	(23)
pET-15b	Inducible expression vector, N-terminal His Tag	Amp^R	Novagen
pSVDA-01	pET-15b encoding His-RNase III	Amp^R	This study
pSVDA-02	pET-15b encoding His-RNase E	Amp^R	This study

samples were collected, mixed with 1 volume of stop solution [10 mM Tris (pH 7.2), 25 mM $NaNO_3$, 5 mM $MgCl_2$, 500 μg/ml chloramphenicol] and harvested by centrifugation (10 min, 6000*g*, 4°C). For stability experiments, rifampicin (500 μg/ml) and nalidixic acid (20 μg/ml) were added to cells grown in LB at 37°C, with agitation, till OD2+6. Incubation was continued and culture aliquots were withdrawn at the time-points indicated in the respective figures. RNA was isolated using the phenol/chlorophorm extraction method, precipitated in ethanol, resuspended in water and quantified on a Nanodrop 1000 machine (NanoDrop Technologies).

For northern blot analysis, 15 μg of total RNA was separated under denaturating conditions either by 8.3 M urea/8% polyacrylamide gel in TBE buffer or by 1.3% agarose MOPS/formaldehyde gel. For polyacrylamide gels, transfer of RNA onto Hybond-N^+ membranes (GE Healthcare) was performed by electroblotting (1 h 50 min, 24 V, 4°C) in TAE buffer. For agarose gels, RNA was transferred to Hybond-N^+ membranes by capillarity using 20 × SSC as transfer buffer. In both cases, RNA was UV cross-linked to the membrane immediately after transfer. Membranes were then hybridized in RapidHyb Buffer (GE Healthcare) at 68°C for riboprobes and 43°C in the case of oligoprobes and DNA probes. After hybridization, membranes were washed as described (17). Signals were visualized by PhosphorImaging (Storm Gel and Blot Imaging System, Amersham Bioscience) and analyzed using the ImageQuant software (Molecular Dynamics).

Hybridization probes

Primers for templates amplification are listed in Supplementary Table S1. Labeling of the riboprobes and oligoprobes was performed as described (17). The riboprobes were obtained using the primer pair pSV-118/pSV-141 for MicA and pSV-142/pSV-143 for *ompA*. The DNA probe for 16S rRNA was generated using the primer pair pSV-144/pSV-145 and 'Amersham Megaprime™ DNA Labeling Systems' (GE Healthcare), according to the supplier instructions.

Construction of recombinant proteins

To overexpress *Salmonella* RNase E and RNase III proteins, the *rne* and *rnc* coding regions were amplified with primer pairs pSV-124/pSV-125 and pSV-129/pSV-130, respectively. The N-terminal region (comprising residues 1–522), corresponding to the catalytic domain of RNase E, was purified. In *E. coli*, the N-terminal half of RNase E (residues 1–498) was reported to be sufficient for the ribonuclease activity (27). The purified PCR products were double digested with BamHI and NdeI and ligated to the pET-15b vector previously digested with the same enzymes, yielding plasmids pSVDA-01 (*rnc*) and pSVDA-02 (*rne*). These plasmids were first cloned into *E. coli* DH5α and were subsequently transformed into BL21(DE3) strain in the case of pSVDA-02, and BL21(DE3) *rnc*105 *rec*A (28) in the case of pSVDA-01 construction. This derivative strain of BL21(DE3), carrying an RNase III mutation, was used because it blocks the autoregulation of *Salmonella* RNase III by the endogenous *E. coli* homologue, resulting in a higher yield of the enzyme upon overexpression. All constructs were confirmed by DNA sequencing at STAB Vida.

Overexpression and purification of *Salmonella* RNase E and RNase III proteins

The BL21 (DE3) strain and derivative, containing the recombinant plasmids of interest, were grown in 100 ml of LB medium supplemented with ampicillin (150 μg/ml) to an optical density at 600 nm of 0.5. At this point, protein expression was induced by addition of 1 mM of IPTG for 3 h at 37°C. Cells were then harvested by centrifugation and the pellets stored at −80°C. The culture pellets expressing RNase III or RNase E were resuspended in 3 ml of Buffer A (20 mM Tris–HCl pH 8, 500 mM NaCl, 20 mM imidazole pH 8). Suspensions were lysed using a French Press at 900 psi in the presence of 0.1 mM of PMSF. After lysis, the crude extracts were treated with 125 U of Benzonase (Sigma) to degrade the nucleic acids and clarified by a 30 min centrifugation at 10 000*g*, 4°C. The histidine tagged recombinant proteins were purified by affinity chromatography, using the ÄKTA FPLC™ System (GE Healthcare). The clarified extracts were loaded into a HisTrap HP Sepharose 1 ml column equilibrated in Buffer A. Protein elution was achieved in buffer A with a linear imidazole gradient (from 20 to 500 mM). The fractions containing mostly the protein of interest, free of contaminants, were pooled. Eluted proteins were buffer exchanged with Desalting Buffer [10 mM Tris–HCl (pH 8), 3 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT] and concentrated by centrifugation at 4°C with Amicon Ultra Centrifugal Filter Devices (Millipore), with a molecular mass cutoff of 10 kDa (RNase III) or 50 kDa (RNase E). Proteins were quantified using the Bradford Method (29) and stored at −20°C in Desalting Buffer containing 50% (v/v) glycerol. The purity of the enzymes was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and revealed >90% homogeneity.

In vitro transcription and activity assays

DNA templates for the *in vitro* transcription were generated by PCR using chromosomal DNA from *S. typhimurium* SL1344 strain. The phage T7 RNA polymerase promoter sequence was included in the forward primer sequences. *micA* was amplified with the primer pair pSV-116/pSV-117, *ompA* with pSV-122/pSV-123 and *lamB* with pSV-120/pSV-121. For the synthesis of the internally labeled 5′ triphosphate MicA, *in vitro* transcription was carried out using the purified PCR product as template in the presence of an excess of [^{32}P]-α-UTP over unlabeled UTP with 'Riboprobe *in vitro* Transcription System' (*Promega*) and T7 RNA polymerase. MicA substrate bearing 5′ monophosphate was obtained by adding an 8-fold excess of GMP over the other ribonucleotides to the *in vitro* transcription reaction. Non-radioactive molecules were transcribed in the same conditions but using equimolar concentrations of all four ribonucleotides. MicA transcripts were purified by electrophoresis on an 8.3 M urea/10% polyacrylamide

gel. The gel slice was crushed and the RNA eluted with elution buffer [3 M ammonium acetate pH 5.2, 1 mM EDTA, 2.5% (v/v) phenol pH 4.3], overnight at room temperature. The RNA was ethanol precipitated and resuspended in RNase free water. For the synthesis of the 5′-end-labeled MicA or *ompA*, *in vitro* transcription was carried out using the corresponding PCR product as template. MicA and *ompA* transcripts were run on a 10 or 6% polyacrylamide gel, respectively, identified by ethidium bromide (EtBr) staining and cut out from the gel. The RNA was eluted from the gel slice as described above. The RNA substrates were end-labeled with [^{32}P]-γ-ATP at 37°C for 1 h, with 10 units of T4 polynucleotide kinase (*Fermentas*) using the supplier exchange buffer and again purified from gel as above. The yield of the labeled substrates (cpm/μl) was determined by scintillation counting.

The hybridization between labeled and unlabeled substrates was always performed in a 1:40 molar ratio in the Tris component of the activity buffer by incubation for 10 min at 80°C, followed by 45 min at 37°C.

The activity assays were done in a final volume of 50 μl containing the activity buffer {for RNase III [30 mM Tris–HCl pH 8, 160 mM NaCl and 0.1 mM DTT] and for RNase E [25 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 60 mM KCl, 100 mM NH_4Cl, 0.1 mM DTT and 5% (v/v) glycerol]} and ~10 000 cpm of substrate. In the case of the activity assays with RNase III, 10 mM of $MgCl_2$ was added to the reaction mixture. As a control, prior to the beginning of each assay an aliquot was taken and was incubated until the end of the assay (without the enzyme). The reactions were started by the addition of the enzyme at a concentration of 500 nM, and further incubated at 37°C in the case of RNase III and 30°C for RNase E (30,31). Samples were withdrawn at the time-points indicated in the respective figures, and the reactions were stopped by the addition of formamide-containing dye supplemented with 10 mM EDTA. Reaction products were resolved in a 7 M urea/15% or 8 % polyacrylamide gel as indicated in the respective figure legends. Signals were visualized by PhosphorImaging and analyzed using ImageQuant software (Molecular Dynamics).

OMPs extraction and analysis

The membrane protein fraction from late stationary phase cultures (OD_{600} of 2 + 6 h) was extracted as described (32). OMPs were analyzed on 4% urea–SDS–12% polyacrylamide gel. Gels were stained overnight with Coomassie Brilliant Blue.

RESULTS

Detection of MicA sense transcripts in an RNase III$^-$ mutant

We have previously studied MicA sRNA turnover in *S. typhimurium* and have analyzed the particular contribution of several RNases to the decay of this sRNA. We have found that the dsRNA-specific endoribonuclease III has a remarkable impact on the stability of MicA sRNA. In the wild-type, MicA sRNA has a half-life of ~6 min (17). In an RNase III$^-$ mutant, there was a dramatic stabilization of the sRNA (no significant decay in >2 h), with the concomitant accumulation of a degradation intermediate, very stable, which was absent in the wild-type (17). In an RNase E mutant MicA was also stabilized, but the small stable intermediate was not detected.

We were interested in clarifying the nature of this small intermediate. For this purpose we have compared the bands pattern of isogenic RNase III$^+$ and RNase III$^-$ strains, by northern blot analysis, with different probes. We have used a probe antisense (AS1) or sense (S1) to the 5′-end of MicA, which corresponds to the region of interaction with its targets (33–35). The same short MicA sRNA stable intermediate of ~45 nt (indicated by an asterisk in the figures) was detected with the antisense probe (AS1) only in the RNase III$^-$ strain (Figure 1A, left panel). When using the MicA sense probe (S1) we have also detected in this mutant a smaller transcript with approximately the same size (Figure 1A, right panel). Both species (sense and antisense) have a remarkably long half-life (see Figure 1B). It was also detected with the sense probe another band with the size corresponding to that of MicA full transcript (74 nt). None of the bands observed with the sense probe were visible in the wild-type or the RNase E mutant (*rne-537*). Moreover, the presence of these 'sense transcripts' is MicA dependent, since they were not detected in an RNase III$^-$/MicA$^-$ strain.

The fact that the smaller transcript is equally present when using a sense or antisense probe and uniquely when RNase III is absent suggests that it is one strand of a stable dsRNA remnant of the MicA-target mRNA paired species. This smaller intermediate probably arises due to the previous activity of other degrading enzyme(s) but only accumulates in the absence of RNase III by virtue of its double stranded character. RNase E is probably a good candidate since the level of the smaller intermediate is decreased in an RNase III$^-$ mutant that is also impaired for degradosome formation - RNase III$^-$/*rne-537* (see Supplementary Figure S1). For instance, cleavage of MicA and *ompA* mRNA (a main target of MicA) by RNase E (33) together with exoribonucleolytic degradation of both RNAs may explain why the antisense and sense transcripts have approximately the same size.

In order to confirm that the smaller transcripts correspond to a stable dsRNA remnant of the MicA-target mRNA paired species, we have used two other MicA sense probes differently located along the MicA transcript (Figure 1C). For each sense probe used, the correspondent antisense probe, complementary to MicA, was also designed. The location of each of the probes in the MicA sequence is indicated in the figure below the respective images. A transcript having the same size was detected whether with the sense probes S1 and S2, or with the corresponding AS1 and AS2 antisense probes. However, we have not obtained any signal when using a sense probe located in the 3′-end of MicA (S3), while the MicA full transcript could still be detected with the corresponding antisense probe (AS3).

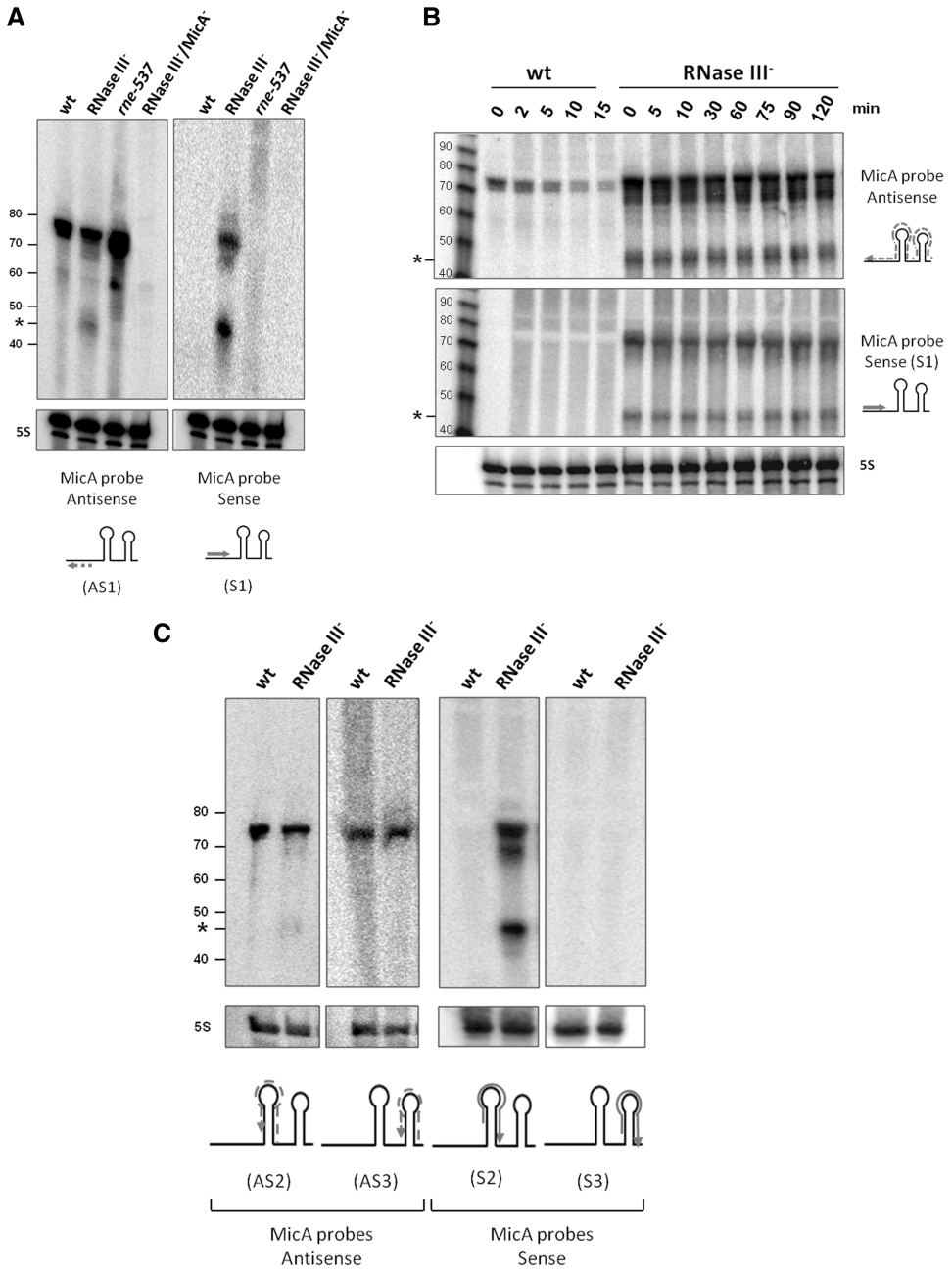

Figure 1. Analysis of MicA sense and antisense species in an RNase III$^-$ mutant. Total cellular RNA was extracted from the *S. typhimurium* strains indicated and analyzed by northern blot. 15 µg of RNA (each lane) were separated on an 8% PAA/8.3 M urea gel. The gel was then blotted to a *Hybond-N*$^+$ membrane and hybridized with the corresponding probes. Details of RNA extraction and northern blot procedure are described in 'Materials and Methods' section. In each case, the membrane was stripped and then probed for 5S rRNA (pSV-139) as loading control. The radiolabeled marker *10bp DNA Step Ladder* (Promega) is on the left side. The respective sizes are represented in nucleotides (full MicA is 74 nt long). The asterisk indicates the fragment that specifically accumulates in RNase III$^-$ strain. The probes used are indicated in the corresponding image. The arrow in each picture indicates the localization and direction of the probes in MicA sRNA: antisense (AS), represented by a dashed arrow; sense (S), represented by a solid arrow. The sequence of the probes is indicated in Supplementary Table S1. (**A**) Total RNA from *S. typhimurium* wild-type and mutant derivatives RNase III$^-$, *rne-537* and RNase III$^-$/MicA$^-$ was hybridized with MicA antisense (AS1) and sense (S1) probes. The double mutant (RNase III$^-$/MicA$^-$) and the RNase E mutant (*rne-537*) were used as controls. (**B**) Comparison of the stability of both MicA sense and antisense species in the absence of RNase III. Total cellular RNA from wild-type and RNase III$^-$ mutant was extracted at the time-points (min) indicated on top, after transcription arrest. RNA samples were analyzed as described above using an antisense probe to the full MicA sequence (upper panel) or a sense probe (lower panel). (**C**) Total RNA from wild-type and RNase III$^-$ mutant strains was hybridized with two other differently located antisense and sense probes, as indicated in the pictures below each image.

These results strongly indicate that the small degradation intermediate should correspond to a remnant of a duplex MicA-target mRNA. The lack of signal when using S3 (located in the 3′-end of MicA) further suggests that the duplex formation is confined to the 5′-end of MicA. This observation is in agreement with previous reports, which indicate that the interaction site is located in the 5′-end of the sRNA, at least for the two known targets of MicA (33–35).

MicA cleavage by RNase III is facilitated by base pairing with its mRNA target(s)

The results presented in Figure 1 suggest that the cleavage of MicA by RNase III occurs in a target- dependent fashion. Taking this into account, together with the fact that RNase III is a double-stranded-specific endoribonuclease, led us to compare *in vitro* the activity of the enzyme both over MicA transcript alone or in complex with its mRNA targets. Until now only two targets for this sRNA have been described in *Salmonella*, *ompA* and *lamB* mRNAs. MicA was reported to act over the translation initiation region of both molecules (33–35). In order to study the activity of *Salmonella* RNase III over MicA, we have cloned and purified the *Salmonella* enzyme as described in Material and Methods. The pure enzyme used in the *in vitro* experiments is shown in Supplementary Figure S2. Activity assays were performed by incubating the purified *Salmonella* RNase III with α^{32}P-labeled MicA alone or in combination with the unlabeled 5′-untranslated region (UTR) of *ompA* or *lamB* mRNAs. Since it has been shown that MicA is also able to bind *ompA* mRNA without the help of Hfq (33), this protein was not included in the activity assays. MicA alone was found to be resistant to RNase III cleavage (Figure 2A). By contrast, in conditions favoring the hybridization of the sRNA transcript with each one of the target molecules, we could see the increasing accumulation of specific reaction products simultaneously with the disappearance of the substrate. This indicates that the formation of the sRNA-target mRNA complex promotes the RNase III cleavage of MicA.

The extension and location of MicA interaction with *ompA* or *lamB* mRNAs has been predicted to be slightly different (33,34). Since RNase III cleaves dsRNA, the different interaction between MicA and the two targets could be in the origin of the distinct cleavage pattern induced by *ompA* or *lamB*. In order to identify the cleavage points generated by RNase III on the MicA-*ompA* and MicA-*lamB* hybrids, *in vitro* assays were performed as described above, but using 5′-end-labeled MicA in combination either with the unlabeled 5′-UTR of *ompA* or *lamB*. The results are shown in Figure 2B. RNase III cleavage generates two main fragments of 22 and 23 nt on MicA-*ompA* hybrid, and 21 and 25 nt on MicA-*lamB*. Since in this experiment MicA was 5′-end-labeled, the size of these fragments indicates the distance from the cleavage point to the 5′-end of MicA. The higher molecular weight bands observed only when MicA was internally labeled (see Figure 2A) correspond to 3′-end fragments, since they are not detected in the cleavage of 5′-end-labeled MicA. A representation of the hybridization regions showing the RNase III cleavage positions in MicA sequence is presented in Figure 2C. All the cleavage positions are located inside the predicted region of interaction with each target, strongly supporting our hypothesis that RNase III is responsible for the coupled MicA-target degradation.

According to our proposal, cleavage of MicA is coupled with the mRNA target cleavage. In this sense the same kind of activity assays were carried out in order to check the direct activity of RNase III over the corresponding region of *ompA* mRNA. For this, the purified *Salmonella* RNase III was incubated with the 5′-end-labeled UTR of *ompA* (172 nt) alone or in combination with unlabeled MicA. As shown in Figure 2D although RNase III is able to cleave free *ompA*, a faster disappearance of the substrate when the hybrid *ompA*-MicA was used indicates that it is cleaved more efficiently. Moreover, the cleavage event gives rise to specific degradation products that were not observed after incubation with *ompA* alone (Figure 2D). Among these products, we could observe the accumulation of fragments in the range of 113–130 nt, which is the expected size of fragments generated by cleavage inside the hybridization region with MicA (see Figure 2C). The other products with a higher molecular weight probably arise due to alterations in the secondary structure of *ompA* after the duplex formation, which could generate a new dsRNA region suitable for RNase III. However, we cannot extrapolate to the *in vivo* situation, since these assays were performed with a truncated version of *ompA*. The ability of RNase III to preferentially cleave the hybrid *ompA*-MicA in the region corresponding to the hybridization between the two molecules is another evidence for the coupled degradation of the target and the sRNA.

Taken together, our results indicate that MicA decay *in vivo* is highly dependent on RNase III and its cleavage by this enzyme *in vitro* is triggered upon base pairing with its target mRNAs.

ompA expression is regulated by RNase III and is dependent on MicA

OmpA is a very abundant porin highly expressed in the exponential phase of growth. In stationary phase MicA is present at high levels and is the principal post-transcriptional downregulator of the *ompA* mRNA (33,35). Since our results indicate that MicA degradation by RNase III is target-dependent and we have observed the concomitant degradation of the *ompA* target mRNA *in vitro*, we analyzed the effect of an RNase III$^-$ mutation on the levels of *ompA* mRNA in stationary phase. The RNase III$^-$ mutant shows an increment of almost 14-fold in *ompA* mRNA level in comparison to the wild-type (Figure 3A). A strong increase in the OmpA protein level was also observed. This suggests that the reduced levels of *ompA* mRNA observed in stationary phase in the wild-type (RNase III$^+$) are probably due to the cleavage and destabilization of the message by RNase III. This cleavage should be suppressed when RNase III is absent. This result strongly indicates that RNase III is

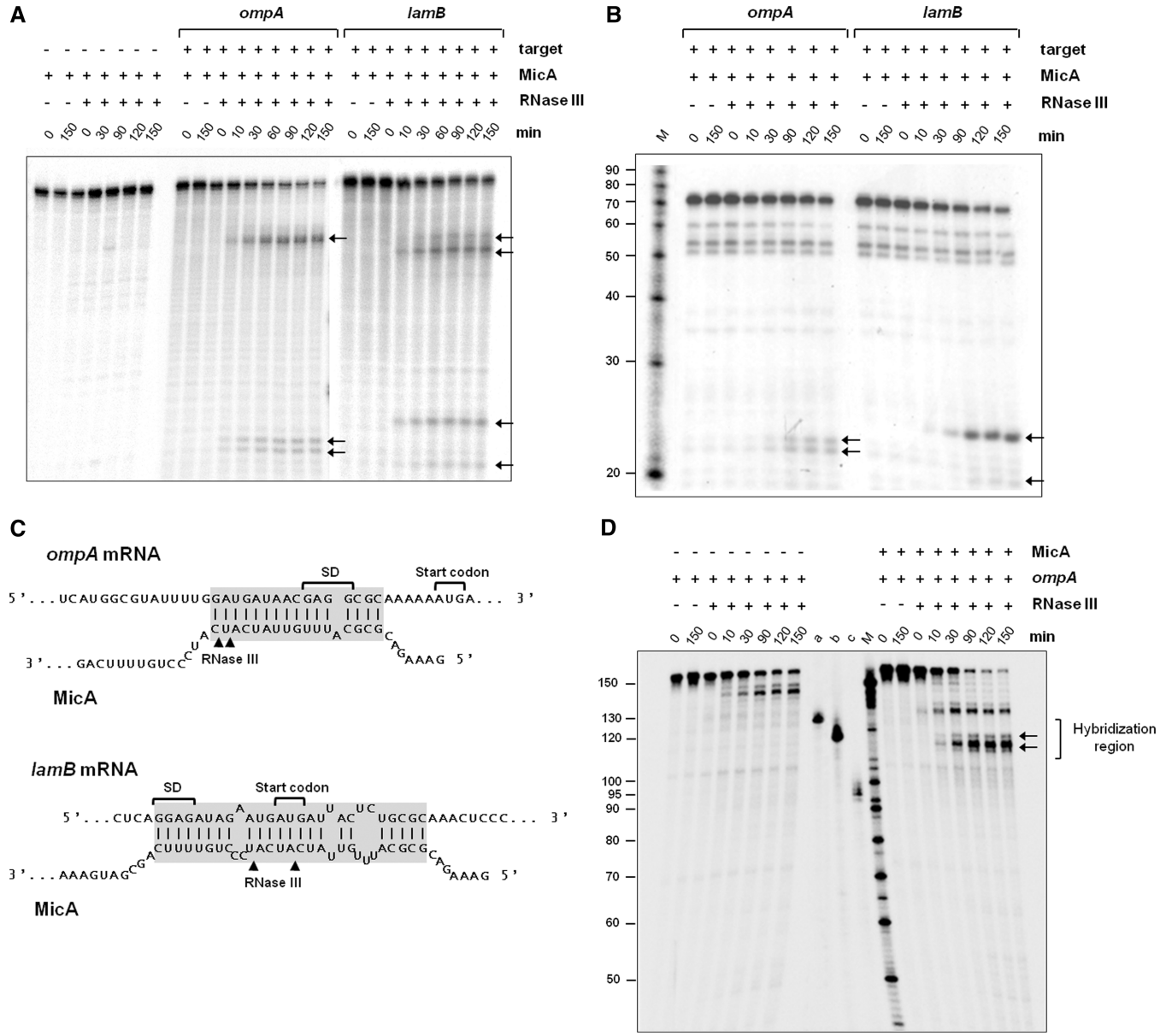

Figure 2. *In vitro* cleavage of sRNA MicA or *ompA* 5′-UTR by endoribonuclease III. The radioactivelly labeled substrate was incubated with 500 nM of *Salmonella* RNase III. Aliquots withdrawn at the time-points indicated above each lane were analyzed on a 7 M urea/15% or 8% PAA gel for MicA or *ompA*, respectively. The first two lanes of each reaction correspond to the controls without the protein at time zero (0) and at the end of the reaction time (150). The radiolabeled Decade Marker RNA (Ambion) is indicated by 'M'. The arrows in the figure indicate specific degradation products. (**A**) Assays performed with internally labeled MicA in the absence (−) (left panel) or in the presence (+) of a molar excess of *ompA (*middle panel) or *lamB* (right panel) unlabeled transcripts (5′-UTR sequence). (**B**) Assays performed with 5′-end-labeled MicA in the presence (+) of a molar excess of *ompA* (left panel) or *lamB* (right panel) unlabeled transcripts (5′-UTR sequence). The bands that are already observed in the absence of the enzyme (control reactions) arise due to the radiolysis of the substrate. (**C**) Proposed interaction regions of *ompA* and *lamB* mRNAs with MicA [adapted from (33)]. The Shine–Dalgarno regions of *ompA* and *lamB* are indicated. The arrows indicate the RNase III cleavage sites on MicA as determined on A and B. (**D**) Assays performed with 5′-end-labeled *ompA* in the absence (−) (on the left) or in the presence (+) (right panel) of a molar excess of unlabeled MicA. On the left side of the marker (M) radiolabeled transcripts of known sizes were included (a) 130 nt; (b) 120 nt and (c) 95 nt. The arrows in the figure indicate the degradation products located inside the hybridization region.

implicated in the degradation of the *ompA* mRNA. On the other hand, the constant levels of OmpC and OmpD between the wild-type and RNase III^{-}, further suggest that RNase III is not involved in the turnover of their messages and that the control of these proteins levels in the cell follows a different pathway. Interestingly, Paperfort *et al.* (4) have shown that MicA sRNA is also not involved in the control of *ompC* or *ompD* levels, while affecting *ompA*.

Since the degradation of both the sRNA MicA and the *ompA* target mRNA is dependent on RNase III, we have checked whether the RNase III regulation of *ompA* expression was also MicA dependent. Therefore, we have analyzed the expression of *ompA* in a MicA^{-} mutant

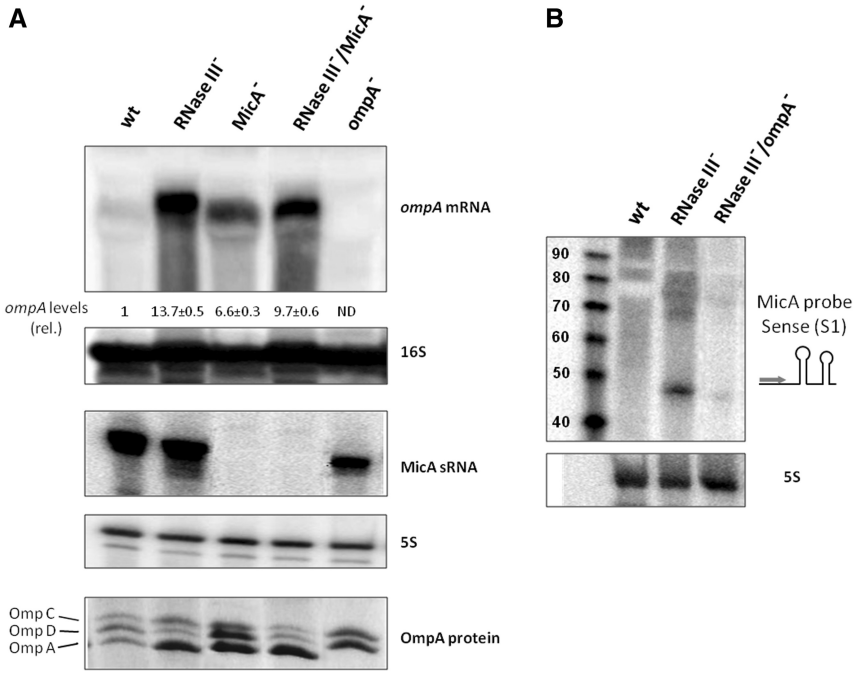

Figure 3. Regulation of *ompA* and MicA expression in different mutant strains. Northern blot and SDS–PAGE analysis of RNA and protein samples extracted from wild-type and mutant strains as indicated on top of each lane. Details of experimental procedures are described in 'Materials and Methods' section. (**A**) (Upper panel) Analysis of steady-state *ompA* mRNA levels by northern blot. 15 μg of RNA (each lane) were resolved in a 1.3% formaldehyde-agarose gel. The gel was then blotted to a Hybond-N^+ membrane and hybridized with the corresponding *ompA* riboprobe. Full-length transcripts were quantified using a Molecular Dynamics PhosphorImager. The amount of RNA found in wild-type was set as one. The ratio between each strain and the wild-type is depicted (relative levels). A representative membrane is shown and values indicated correspond to the average of several northern blot experiments with RNAs from at least two independent extractions. The membrane was stripped and then probed for 16S rRNA as loading control. (ND) Non-detectable. (Middle panel) MicA sRNA levels analysis by northern blot. 15 μg of RNA from the same mutants were separated on an 8% PAA/8.3 M urea. The gel was then blotted to a Hybond-N^+ membrane and hybridized with the corresponding MicA riboprobe. The membrane was stripped and then probed for 5S rRNAs, as loading control. (Lower pannel) Outer membrane protein fraction analysis by 4% urea–SDS–12% polyacrylamide gel electrophoresis. The positions of the OmpC, OmpD and OmpA bands are indicated. An $OmpA^-$ mutant was used as control. (**B**) Comparison of the levels of MicA sense species on the wild-type, RNase III^- and the double RNase $III^-/OmpA^-$ strains. The experimental procedure was similar to the one described in (A). The arrow on MicA sRNA picture indicates the localization and direction of the probe (S1). Loading control of the RNA was done with 5S rRNA probe and is represented below. Sizes were estimated using the radiolabeled 10bp DNA Step Ladder (Promega), on the left side of the membrane.

strain. In stationary phase MicA basepairs with the 5′-UTR of *ompA* preventing ribosome binding and destabilizing the entire *ompA* mRNA (33,35). Accordingly, when the regulator is absent ($MicA^-$ mutant) the levels of *ompA* mRNA should be elevated. We observed an increase of about 7-fold in *ompA* mRNA levels when MicA is absent (Figure 3A). This result confirms that the control of *ompA* mRNA levels is dependent on MicA. However, the fact that in the absence of RNase III *ompA* mRNA levels are still higher than in the $MicA^-$ mutant indicates that RNase III may also have a role in *ompA* expression by an alternative pathway not involving MicA. In fact we show that RNase III is also able to cleave *ompA in vitro* in the absence of MicA. Additionally *ompA* mRNA may also be under the control of another sRNA in an RNase III dependent way.

If RNase III and MicA affect the *ompA* message through the same regulatory pathway, the combined absence of both would not result in a cumulative effect. In order to clarify this, we have constructed and tested the effect of the double mutant $RNase\ III^-/MicA^-$ on the *ompA* mRNA levels. As shown in Figure 3A, in the double $RNase\ III^-/MicA^-$ mutant the *ompA* levels are reduced in comparison with the RNase III^- single mutant, demonstrating that MicA and RNase III act over *ompA* message through a common pathway.

Detection of the 'sense transcripts' in the RNase III^- mutant depends on *ompA*

Taken together, the results presented here point out that *ompA* is subjected to MicA-coupled degradation by RNase III. Since we had indications that the 'sense transcripts' detected in the RNase III^- mutant are remnants of the sRNA-target complex (see Figure 1), we have investigated if these 'sense-transcripts' corresponded to *ompA* mRNA fragments. Indeed, in the absence of both RNase III and *ompA*, the levels of these 'sense transcripts' are largely reduced when compared with those of the single RNase III^- mutant (Figure 3B). This means that the detection of these 'sense transcripts' is related with the presence of *ompA*, strongly suggesting that this mRNA might be one of the targets degraded by RNase III in conjunction with MicA.

The fact that the 'sense transcripts' are still detectable in the absence of *ompA* indicates that this mRNA might not be the only candidate for the MicA-coupled degradation. However, in a LamB$^-$ mutant (the other known target of MicA) we did not observe, under our experimental conditions, a significant alteration in the level of the 'sense transcripts' (data not shown). Furthermore, in the absence of both *ompA* and *lamB* targets, the 'sense transcripts' could still be slightly observed (data not shown), indicating that other MicA targets subjected to the same type of regulation should exist in the cell.

RNase E cleaves 'free MicA' sRNA *in vitro*

We have demonstrated that the sRNA MicA degradation is influenced by RNase III. However, this seems to happen only in the presence of the target mRNA. As we have shown *in vitro*, the enzyme was not able to cleave MicA alone. Thus, the question of how is free MicA degraded remains to be answered. *In vivo* experiments have shown a large impact of an RNase E mutant on the levels and stability of full MicA sRNA (17). However, these results concern studies undertaken with the *rne-537* mutant derivative (17). Since this mutant only prevents degradosome formation, without totally abolishing the enzyme activity, we were also interested in clarifying the role of the catalytic activity of RNase E on the decay of MicA. Moreover, it has been shown that -A/U rich sequences together with adjacent stem-loop structures can comprise recognition sites for RNase E (36,37). The sequence of the sRNA MicA matches these characteristics. Therefore, we have analyzed the ability of this endoribonuclease to cleave MicA sRNA transcript, *in vitro*. For this purpose we have cloned and purified the amino-terminal region of *Salmonella* RNase E. The homologous region in *E. coli* RNase E is known to be responsible for the catalytic activity of the enzyme (27). The results of the purification of the N-terminal segment of *Salmonella* RNase E are shown in Supplementary Figure S2. *In vitro* assays with the purified protein were performed over uniformly labeled MicA transcript. It was seen before that RNase E preferentially cleaves RNAs with a 5′ monophosphate group over those endowed with a 5′ triphosphate (36,38–40). Thus, in the activity assays we have used as substrate both the monophosphate and the triphosphate MicA transcripts. Our results show that RNase E is able to cleave both substrates *in vitro* (Figure 4), though the efficiency of cleavage was superior over monophosphorylated MicA. This is in agreement with the recent report that *E. coli* RNase E is also active over some triphosphate substrates (41).

We have previously shown that in cells in which the degradosome scaffold of RNase E was deleted the degradation of MicA is slower (>4-fold stabilization) (17). This suggests that, *in vivo*, RNase E may need the cooperation of other degradosome components in the decay of this transcript. Indeed it was previously shown in *E. coli* and *Salmonella* that the absence of PNPase, the exoribonucleolytic component of the degradosome, has a remarkable impact over the stability of MicA sRNA (16,17). However, the high ability of RNase E to cleave MicA *in vitro* indicates that the enzyme *per se* should importantly contribute for the *in vivo* degradation of free MicA.

DISCUSSION

Stress conditions that unbalance OMP levels activate the σ^E response, a complex set of changes normally devoted to protect the cell envelope from environmental challenges (42). The transcription factor σ^E triggers the synthesis of the sRNAs that control OMP levels (4,6). Upon downregulation of OMPs and the relief of membrane stress, the high sRNA levels have to be brought back to normal amounts. MicA sRNA is a σ^E-dependent porin downregulator whose transcription is activated in stationary-phase (4,6,9). Under this context, we were interested in studying the regulation of MicA cellular levels and determining the enzymes involved in this process.

MicA was previously found to be highly stabilized in cells lacking a functional RNase III (17). However, RNase III is not able to cleave MicA *in vitro*, suggesting that MicA alone is not a substrate for this enzyme. Indeed, we demonstrate that RNase III is only able to cleave MicA *in vitro* when it base pairs with its target(s). RNase III is a specific double stranded RNA endoribonuclease, which plays multiple roles in the processing of rRNA and mRNA (43) and its activity has also been demonstrated over several sRNA-target complexes formed by *cis*-antisense sRNAs (44–47). In these complexes, there is a perfect complementarity between the RNA partners, which constitutes a preferred substrate for RNase III, and avoids the need for Hfq. The limited complementary between *trans*-encoded sRNAs and their

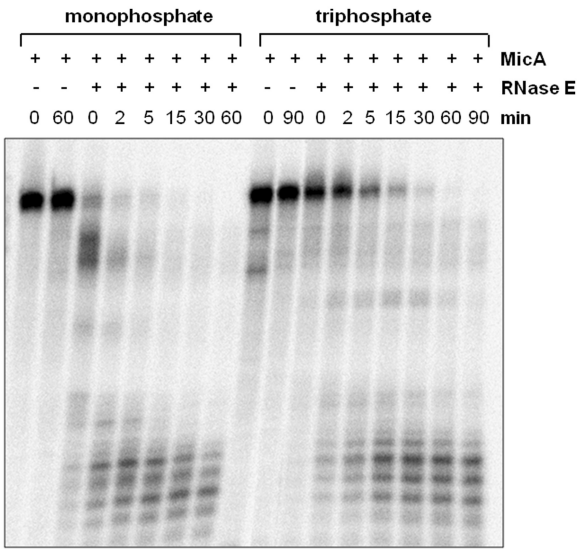

Figure 4. *In vitro* study of MicA sRNA cleavage by RNase E. α-^{32}P-labeled MicA transcript, 5′ monophosphate (left panel) or 5′ triphosphate (right panel), was incubated with 500 nM of purified *Salmonella* RNase E (residues 1–522) at 30°C. Aliquots withdrawn at the time-points indicated above each lane were analyzed on a 15% PAA/7M urea gel. The two first lanes of each reaction correspond to the controls without the protein both withdrawn at time zero and at the end of the reaction time.

targets typically requires the help of the bacterial RNA chaperone Hfq. The *trans*-encoded sRNA MicA was shown to be dependent on Hfq both for stability and target degradation (17,33,35). It is generally assumed that Hfq binds both the regulator and the target RNA, favoring their interaction. Moreover, Hfq enhances the stability of many sRNAs *in vivo*, by protecting them from degradation (48). Curiously, IstR-1 from *E. coli* and RNAIII from *Staphylococcus aureus* are two *trans*-encoded sRNAs that act independently of Hfq and were also seen to be cleaved by RNase III in a target-coupled mechanism (13,49–51). Here we describe, in *Salmonella*, the first example of a system controlled by a Hfq-dependent *trans*-sRNA that involves the coupled degradation of the sRNA-target mRNA by RNase III.

In RNase III$^-$ cells, besides the high stabilization of MicA, a very stable smaller degradation intermediate is also observed. In agreement with the *in vitro* results, we have obtained several indications that this degradation intermediate corresponds to a remnant of a dsRNA complex formed by MicA and its target(s): (i) It is only detected in the RNase III$^-$ mutant (deficient for dsRNA degradation), where it is extremely stable by virtue of its double stranded character. When RNase III is present, dsRNA complexes are cleaved to products that are either further degraded or too small to be detected by northern blot; (ii) This small intermediate is visible with both antisense MicA probes and sense probes complementary to the targets and (iii) The level detected with sense probes is highly reduced in the absence of OmpA (a main MicA target). Thus, this remnant species should indicate the region of interaction between MicA and its targets. Since a strong signal is detected with the 5′ probes and no signal at all is obtained with the probes located in the 3′-end, this region corresponds to the 5′ half of MicA. In fact, it is known that MicA interacts through its 5′-end sequence with its two targets described till now (33–35). Accordingly, the RNase III cleavage sites determined *in vitro* on both MicA-*ompA* and MicA-*lamB* hybrids are located in the 5′ half of MicA, inside the respective predicted hybridization region, which strongly correlates with the *in vivo* observations. This result is further confirmed by the fact that RNase III also cleaves the *ompA* 5′-UTR inside the same region, demonstrating that both molecules are cleaved together.

In the absence of both *ompA* and RNase III the level of the 'sense transcripts' is strongly decreased (Figure 3B). The fact that these bands are still visible, despite at very low levels, may be related with the formation of complexes between MicA and other target(s), whose degradation should also be RNase III-dependent. We demonstrate that *in vitro* RNase III is also able to cleave the complex MicA-*lamB*. However, *in vivo*, whether in the absence of *lamB* or of both *lamB* and *ompA* we could still detect the sense transcripts referred above (data not shown). This means that probably besides *lamB* mRNA other *Salmonella* MicA targets (not yet identified) may exist in stationary-phase. In *E. coli,* expression of *phoPQ* has recently been shown to be repressed by MicA upon activation of σ^E (52). In fact, MicA is a *trans*-encoded sRNA highly conserved in Enterobacteriacea (53). *Trans*-encoded sRNAs generally establish short and imperfect interactions with its mRNA targets (~10–25 nt) (11,54), allowing the regulation of multiple targets by the same sRNA. For example, RybB sRNA controls more than 17 mRNAs, 10 of which encode OMPs, including *ompA*.

Upon MicA accumulation in stationary phase, the sRNA binds to *ompA* mRNA blocking ribosome binding and translation initiation. This releases the mRNA from the 'protection' by the ribosomes and leads to degradation of the ribosome-free mRNA by the concerted action of endo- and exoribonucleases (19). In line with previous-work (55,56), RNase E is thought to be the endoribonuclease responsible for the decay of *ompA* mRNA after the blockage of ribosome loading caused by MicA binding (33,35). In this report, we show that additionally the binding of MicA to *ompA* renders both RNAs susceptible to RNase III cleavage. We have demonstrated that this endoribonuclease is essential for *ompA* repression and, in agreement with previous studies (33,35), we observed a relieve of *ompA* repression in the absence of MicA. Both effects were seen to occur in a concerted way. The RNase III pathway has the advantage of simultaneously controlling the levels of the sRNA, whose function after the repression of the target will no longer be necessary in the cell. In addition RNase III cleavage makes the repression irreversible. From a physiological point of view, the existence of two distinct pathways may enhance the cell response in stress conditions allowing a fine-tuned balance of OmpA levels needed to keep the envelop integrity. Moreover by having two alternative degradation pathways the cell warrants the metabolism of molecules no longer needed. This may be crucial in stationary phase, which is characterized by limited resources.

Interestingly, the *ompA* levels in the double mutant (RNase III$^-$/MicA$^-$) are not restored to those observed in the MicA$^-$ mutant. This can be due to a direct effect of RNase III over *ompA*. Indeed we show that *in vitro* RNase III is able to cleave *ompA* even in the absence of the sRNA. Alternatively, other(s) player(s) can be involved in the RNase III-mediated regulation of *ompA*. At least two other *trans*-encoded regulatory sRNAs (RseX and RybB) have been described as additional *ompA* regulators (4,5).

We have just described a pathway for degradation of MicA sRNA that involves target binding and is dependent on RNase III. Our results show that RNase III cleaves MicA when hybridized with the targets. The question then arises how the levels of free MicA are brought back to normal when the cell no longer needs it. Our previous results *in vivo*, showed a high stabilization of MicA in an RNase E deletion mutant lacking the C-terminal region (17). This may suggest that RNase E needs the cooperation of other degradosome components in the degradation of MicA. In fact, we have previously shown that PNPase, the exonucleolytic component of the degradosome, has a great impact over the stability of this sRNA in *Salmonella*. According to the results presented here, the catalytic domain of RNase E also shows, *in vitro*, a high efficiency in the cleavage of this

sRNA. This single stranded endoribonuclease seems to play an important role in the regulation of the abundance of free MicA.

For each sRNA the characterization of its turnover has to be analyzed from two different perspectives: the independent, and the dependent of target interaction. The later can be similar or not, whether the sRNA decay is influenced or not by the respective target(s). Taken together, the results presented in this study indicate the existence of two different pathways for MicA sRNA turnover, each one involving a specific endoribonuclease. According to the model proposed in Figure 5, when MicA is free, RNase E seems to take the control by efficiently degrading the sRNA. However, if MicA is interacting with the targets the target-dependent pathway of degradation predominates. This mechanism involves a double stranded endoribonuclease that is able to degrade both the target and the sRNA, simultaneously.

Cleavage by RNase III within the sRNA–mRNA duplex and the subsequent decay of the mRNA intermediate by the cell machinery could rather resemble the RNAi scenario in eukaryotic organisms. RNase III-like enzymes are known to have a pivotal role in eukaryotic small non-coding RNA function and biogenesis (19). Hence, it is not surprising that RNase III would also be a main player in the control of prokaryotic sRNA expression and function, broadening the enzyme's global role in the regulation of gene expression.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

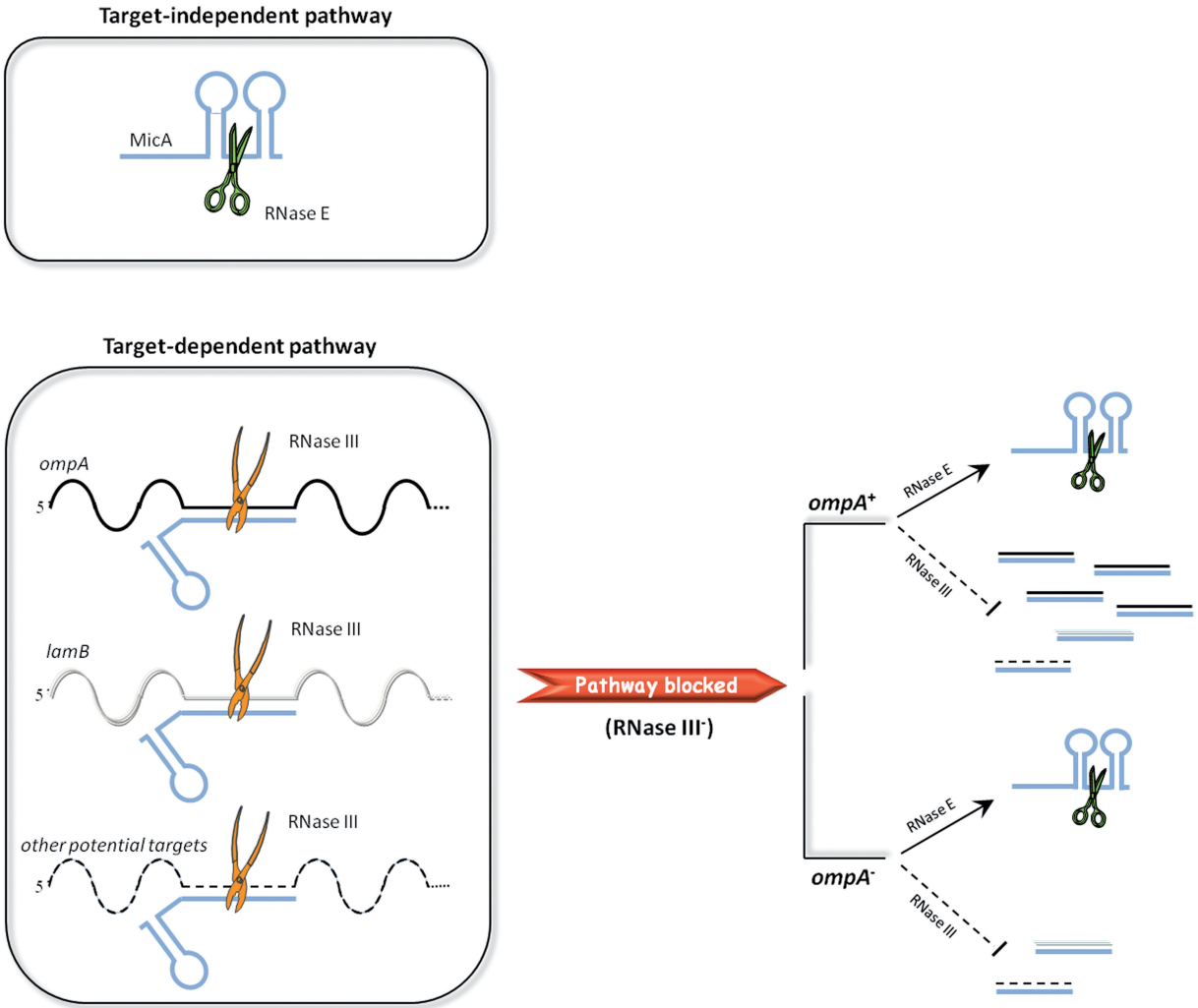

Figure 5. Schematic representation of the two degradation pathways followed by MicA. RNase E and RNase III are represented by scissors and pliers, respectively. The two different pathways for MicA degradation are shown on the left side. The possible associations of MicA with its targets are also depicted. In the wild-type, MicA and the targets should be fully degraded as a result of both degradation pathways in cooperation with the exoribonucleolytic activity. In the RNase III^{-} mutant, the MicA-target dependent degradation by RNase III is blocked. As a result, some degradation intermediates are stabilized and can be detected, namely the target and MicA strands that have interacted but could not be cleaved by RNase III. *ompA* being the main target of MicA, its species are over-represented. When additionally the *ompA* target mRNA is absent, the respective degradation intermediate is no longer present in the cell and, as a consequence, there is a reduced level of transcripts detected with probes complementary to MicA-targets.

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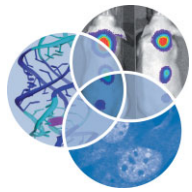
REFERENCES

1. Mizuno,T., Chou,M.Y. and Inouye,M. (1984) A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). *Proc. Natl Acad. Sci. USA*, **81**, 1966–1970.
2. Livny,J. and Waldor,M.K. (2007) Identification of small RNAs in diverse bacterial species. *Curr. Opin. Microbiol.*, **10**, 96–101.
3. Guillier,M. and Gottesman,S. (2006) Remodelling of the *Escherichia coli* outer membrane by two small regulatory RNAs. *Mol. Microbiol.*, **59**, 231–247.
4. Papenfort,K., Pfeiffer,V., Mika,F., Lucchini,S., Hinton,J.C. and Vogel,J. (2006) SigmaE-dependent small RNAs of *Salmonella* respond to membrane stress by accelerating global *omp* mRNA decay. *Mol. Microbiol.*, **62**, 1674–1688.
5. Douchin,V., Bohn,C. and Bouloc,P. (2006) Down-regulation of porins by a small RNA bypasses the essentiality of the regulated intramembrane proteolysis protease RseP in *Escherichia coli*. *J. Biol. Chem.*, **281**, 12253–12259.
6. Johansen,J., Rasmussen,A.A., Overgaard,M. and Valentin-Hansen,P. (2006) Conserved small non-coding RNAs that belong to the sigmaE regulon: role in down-regulation of outer membrane proteins. *J. Mol. Biol.*, **364**, 1–8.
7. Alba,B.M. and Gross,C.A. (2004) Regulation of the *Escherichia coli* sigma-dependent envelope stress response. *Mol. Microbiol.*, **52**, 613–619.
8. Ruiz,N. and Silhavy,T.J. (2005) Sensing external stress: watchdogs of the *Escherichia coli* cell envelope. *Curr. Opin. Microbiol.*, **8**, 122–126.
9. Figueroa-Bossi,N., Lemire,S., Maloriol,D., Balbontin,R., Casadesus,J. and Bossi,L. (2006) Loss of Hfq activates the sigmaE-dependent envelope stress response in *Salmonella enterica*. *Mol. Microbiol.*, **62**, 838–852.
10. Vogel,J. and Wagner,E.G. (2007) Target identification of small noncoding RNAs in bacteria. *Curr. Opin. Microbiol.*, **10**, 262–270.
11. Waters,L.S. and Storz,G. (2009) Regulatory RNAs in bacteria. *Cell*, **136**, 615–628.
12. Massé,E., Escorcia,F.E. and Gottesman,S. (2003) Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. *Genes Dev.*, **17**, 2374–2383.
13. Vogel,J., Argaman,L., Wagner,E.G. and Altuvia,S. (2004) The small RNA IstR inhibits synthesis of an SOS-induced toxic peptide. *Curr. Biol.*, **14**, 2271–2276.
14. Afonyushkin,T., Vecerek,B., Moll,I., Blasi,U. and Kaberdin,V.R. (2005) Both RNase E and RNase III control the stability of *sodB* mRNA upon translational inhibition by the small regulatory RNA RyhB. *Nucleic Acids Res.*, **33**, 1678–1689.
15. Morita,T., Maki,K. and Aiba,H. (2005) RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs. *Genes Dev.*, **19**, 2176–2186.
16. Andrade,J.M. and Arraiano,C.M. (2008) PNPase is a key player in the regulation of small RNAs that control the expression of outer membrane proteins. *RNA*, **14**, 543–551.
17. Viegas,S.C., Pfeiffer,V., Sittka,A., Silva,I.J., Vogel,J. and Arraiano,C.M. (2007) Characterization of the role of ribonucleases in *Salmonella* small RNA decay. *Nucleic Acids Res.*, **35**, 7651–7664.
18. Viegas,S.C. and Arraiano,C.M. (2008) Regulating the regulators: How ribonucleases dictate the rules in the control of small non-coding RNAs. *RNA Biol.*, **5**, 230–243.
19. Arraiano,C.M., Andrade,J.M., Domingues,S., Guinote,I.B., Malecki,M., Matos,R.G., Moreira,R.N., Pobre,V., Reis,F.P., Saramago,M. *et al.* (2010) The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol. Rev.*, **34**, 883–923.
20. Carpousis,A.J., Luisi,B.F. and McDowall,K.J. (2009) Endonucleolytic initiation of mRNA decay in *Escherichia coli*. *Prog. Mol. Biol. Transl. Sci.*, **85**, 91–135.
21. Conrad,C. and Rauhut,R. (2002) Ribonuclease III: new sense from nuisance. *Int. J. Biochem. Cell Biol.*, **34**, 116–129.
22. Andrade,J.M., Pobre,V., Silva,I.J., Domingues,S. and Arraiano,C.M. (2009) The role of 3′-5′ exoribonucleases in RNA degradation. *Prog. Mol. Biol. Transl. Sci.*, **85**, 187–229.
23. Kaberdin,V.R. and Blasi,U. (2006) Translation initiation and the fate of bacterial mRNAs. *FEMS Microbiol. Rev.*, **30**, 967–979.
24. Datsenko,K.A. and Wanner,B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl Acad. Sci. USA*, **97**, 6640–6645.
25. Schmieger,H. (1971) The fate of the bacterial chromosome in P22-infected cells of *Salmonella typhimurium*. *Mol. Gen. Genet.*, **110**, 238–244.
26. Mattatall,N.R. and Sanderson,K.E. (1998) RNase III deficient *Salmonella typhimurium* LT2 contains intervening sequences (IVSs) in its 23S rRNA. *FEMS Microbiol. Lett.*, **159**, 179–185.
27. McDowall,K.J. and Cohen,S.N. (1996) The N-terminal domain of the *rne* gene product has RNase E activity and is non-overlapping with the arginine-rich RNA-binding site. *J. Mol. Biol.*, **255**, 349–355.
28. Amarasinghe,A.K., Calin-Jageman,I., Harmouch,A., Sun,W. and Nicholson,A.W. (2001) *Escherichia coli* ribonuclease III: affinity purification of hexahistidine-tagged enzyme and assays for substrate binding and cleavage. *Methods Enzymol.*, **342**, 143–158.
29. Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.
30. Chelladurai,B.S., Li,H. and Nicholson,A.W. (1991) A conserved sequence element in ribonuclease III processing signals is not required for accurate *in vitro* enzymatic cleavage. *Nucleic Acids Res.*, **19**, 1759–1766.
31. Jiang,X., Diwa,A. and Belasco,J.G. (2000) Regions of RNase E important for 5′-end-dependent RNA cleavage and autoregulated synthesis. *J. Bacteriol.*, **182**, 2468–2475.
32. Matsuyama,S., Inokuchi,K. and Mizushima,S. (1984) Promoter exchange between *ompF* and *ompC*, genes for osmoregulated major outer membrane proteins of *Escherichia coli* K-12. *J. Bacteriol.*, **158**, 1041–1047.
33. Udekwu,K.I., Darfeuille,F., Vogel,J., Reimegard,J., Holmqvist,E. and Wagner,E.G. (2005) Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. *Genes Dev.*, **19**, 2355–2366.
34. Bossi,L. and Figueroa-Bossi,N. (2007) A small RNA downregulates LamB maltoporin in *Salmonella*. *Mol. Microbiol.*, **65**, 799–810.
35. Rasmussen,A.A., Eriksen,M., Gilany,K., Udesen,C., Franch,T., Petersen,C. and Valentin-Hansen,P. (2005) Regulation of *ompA* mRNA stability: the role of a small regulatory RNA in growth phase-dependent control. *Mol. Microbiol.*, **58**, 1421–1429.
36. Mackie,G.A. (1998) Ribonuclease E is a 5′-end-dependent endonuclease. *Nature*, **395**, 720–723.
37. Kaberdin,V.R., Walsh,A.P., Jakobsen,T., McDowall,K.J. and von Gabain,A. (2000) Enhanced cleavage of RNA mediated by an interaction between substrates and the arginine-rich domain of *E. coli* ribonuclease E. *J. Mol. Biol.*, **301**, 257–264.

38. Lin-Chao,S. and Cohen,S.N. (1991) The rate of processing and degradation of antisense RNAI regulates the replication of ColE1-type plasmids *in vivo*. *Cell*, **65**, 1233–1242.
39. Mackie,G.A. (2000) Stabilization of circular *rpsT* mRNA demonstrates the 5′-end dependence of RNase E action *in vivo*. *J. Biol. Chem.*, **275**, 25069–25072.
40. Celesnik,H., Deana,A. and Belasco,J.G. (2007) Initiation of RNA decay in *Escherichia coli* by 5′ pyrophosphate removal. *Mol. Cell*, **27**, 79–90.
41. Kime,L., Jourdan,S.S., Stead,J.A., Hidalgo-Sastre,A. and McDowall,K.J. (2010) Rapid cleavage of RNA by RNase E in the absence of 5′ monophosphate stimulation. *Mol. Microbiol.*, **76**, 590–604.
42. Rowley,G., Spector,M., Kormanec,J. and Roberts,M. (2006) Pushing the envelope: extracytoplasmic stress responses in bacterial pathogens. *Nat. Rev. Microbiol.*, **4**, 383–394.
43. Nicholson,A.W. (1999) Function, mechanism and regulation of bacterial ribonucleases. *FEMS Microbiol. Rev.*, **23**, 371–390.
44. Simons,R.W. and Kleckner,N. (1988) Biological regulation by antisense RNA in prokaryotes. *Annu. Rev. Genet.*, **22**, 567–600.
45. Krinke,L. and Wulff,D.L. (1987) OOP RNA, produced from multicopy plasmids, inhibits lambda *cII* gene expression through an RNase III-dependent mechanism. *Genes Dev.*, **1**, 1005–1013.
46. Jerome,L.J., van Biesen,T. and Frost,L.S. (1999) Degradation of FinP antisense RNA from F-like plasmids: the RNA-binding protein, FinO, protects FinP from ribonuclease E. *J. Mol. Biol.*, **285**, 1457–1473.
47. Gerdes,K., Nielsen,A., Thorsted,P. and Wagner,E.G. (1992) Mechanism of killer gene activation. Antisense RNA-dependent RNase III cleavage ensures rapid turn-over of the stable *hok, srnB* and *pndA* effector messenger RNAs. *J. Mol. Biol.*, **226**, 637–649.
48. Storz,G., Altuvia,S. and Wassarman,K.M. (2005) An abundance of RNA regulators. *Annu. Rev. Biochem.*, **74**, 199–217.
49. Darfeuille,F., Unoson,C., Vogel,J. and Wagner,E.G. (2007) An antisense RNA inhibits translation by competing with standby ribosomes. *Mol. Cell*, **26**, 381–392.
50. Huntzinger,E., Boisset,S., Saveanu,C., Benito,Y., Geissmann,T., Namane,A., Lina,G., Etienne,J., Ehresmann,B., Ehresmann,C. *et al.* (2005) *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate *spa* gene expression. *EMBO J.*, **24**, 824–835.
51. Boisset,S., Geissmann,T., Huntzinger,E., Fechter,P., Bendridi,N., Possedko,M., Chevalier,C., Helfer,A.C., Benito,Y., Jacquier,A. *et al.* (2007) *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. *Genes Dev.*, **21**, 1353–1366.
52. Coornaert,A., Lu,A., Mandin,P., Springer,M., Gottesman,S. and Guillier,M. (2010) MicA sRNA links the PhoP regulon to cell envelope stress. *Mol. Microbiol.*, **76**, 467–479.
53. Vogel,J. and Papenfort,K. (2006) Small non-coding RNAs and the bacterial outer membrane. *Curr. Opin. Microbiol.*, **9**, 605–611.
54. Kawamoto,H., Koide,Y., Morita,T. and Aiba,H. (2006) Base-pairing requirement for RNA silencing by a bacterial small RNA and acceleration of duplex formation by Hfq. *Mol. Microbiol.*, **61**, 1013–1022.
55. Melefors,O. and von Gabain,A. (1988) Site-specific endonucleolytic cleavages and the regulation of stability of *E. coli ompA* mRNA. *Cell*, **52**, 893–901.
56. Vytvytska,O., Jakobsen,J.S., Balcunaite,G., Andersen,J.S., Baccarini,M. and von Gabain,A. (1998) Host factor I, Hfq, binds to *Escherichia coli ompA* mRNA in a growth rate-dependent fashion and regulates its stability. *Proc. Natl Acad. Sci. USA*, **95**, 14118–14123.
57. Hoiseth,S.K. and Stocker,B.A. (1981) Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature*, **291**, 238–239.
58. Studier,F.W. and Moffatt,B.A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.*, **189**, 113–130.

Advanced Review



Importance and key events of prokaryotic RNA decay: the ultimate fate of an RNA molecule

Inês Jesus Silva, Margarida Saramago, Clémentine Dressaire, Susana Domingues, Sandra Cristina Viegas and Cecília Maria Arraiano*

RNAs are important effectors in the process of gene expression. In bacteria, constant adaptation to environmental demands is accompanied by a continual adjustment of transcripts' levels. The cellular concentration of a given RNA is the result of the balance between its synthesis and degradation. RNA degradation is a complex process encompassing multiple pathways. Ribonucleases (RNases) are the enzymes that directly process and degrade the transcripts, regulating their amounts. They are also important in quality control of RNAs by detecting and destroying defective molecules. The rate at which RNA decay occurs depends on the availability of ribonucleases and their specificities according to the sequence and/or the structural elements of the RNA molecule. Ribosome loading and the 5′-phosphorylation status can also modulate the stability of transcripts. The wide diversity of RNases present in different microorganisms is another factor that conditions the pathways and mechanisms of RNA degradation. RNases are themselves carefully regulated by distinct mechanisms.

Several other factors modulate RNA degradation, namely polyadenylation, which plays a multifunctional role in RNA metabolism. Additionally, small non-coding RNAs are crucial regulators of gene expression, and can directly modulate the stability of their mRNA targets. In many cases this regulation is dependent on Hfq, an RNA binding protein which can act in concert with polyadenylation enzymes and is often necessary for the activity of sRNAs.

All of the above-mentioned aspects are discussed in the present review, which also highlights the principal differences between the RNA degradation pathways for the two main Gram-negative and Gram-positive bacterial models. *WIREs RNA* 2011 2 818–836 DOI: 10.1002/wrna.94

INTRODUCTION

Bacteria have evolved complex regulatory networks in order to adjust their physiology and survive in face of constantly changing environmental conditions. Maintenance of cellular functions relies on the proper expression of genetic information, in which the RNA molecules play a key role. Among RNAs, messenger RNAs (mRNA) constitute the molecular link between genes and proteins. Because the cellular concentration of a given transcript depends on the rates of its synthesis and degradation, both transcription and degradation control the levels of each protein in the cell. Regulating gene expression at the messenger level is of utmost importance for guaranteeing versatility in the context of the small genome size found in prokaryotes where transcription and translation are coupled. Survival and development in challenging growth conditions require a rapid adaptation of gene expression. In this regard, control of transcription

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and degradation of mRNA requires less energy and proceeds much faster than translation and protein degradation processes.[1] The rate of turnover is not related to the length of the gene; the stability of the gene transcripts seems to be regulated by determinants located in specific mRNA segments and the segments that decay more rapidly can be located anywhere in the mRNA. Moreover, distinct processing may confer differential stability upon the fragments of a polycistronic transcript. The complexity of the RNA degradation process was further revealed with the discovery of non-coding RNAs (sRNAs). Previously overlooked, sRNAs are now known to exert significant regulatory effect on gene expression, and have thus attracted increasing interest due to their regulatory functions, as well as their role in bacterial adaptation and virulence.[2,3] Furthermore, the regulation of their cellular levels constitutes an upstream control of gene expression.

In this article, we present the current knowledge on prokaryotic RNA degradation mechanisms. We discuss several questions like: which are the main players involved? What is their mode of action? Are there any differences between bacterial species? After a brief overview of the genome-wide studies of RNA decay, prokaryotic RNA degradation mechanisms are described. We also look into the RNA features and the structural details of ribonucleases, the enzymes that process and degrade RNA. Finally, we focus on additional factors which have an impact on RNA stability. Together, these key events determine the ultimate fate of an RNA molecule.

RNA DECAY: GLOBAL APPROACHES

The development of transcriptome analysis has enabled the monitoring of intracellular RNA levels at the genomic scale. The rate of degradation of a given RNA can be estimated by determining its half-life in the cell. Combining inhibition of transcription with microarray technology has proven to be a powerful tool for assessing genome-wide mRNA decay. Most of these global studies in microorganisms have been carried out on the model yeast *Saccharomyces cerevisiae*.[4–6] mRNA half-lives ranging from 3 to more than 100 min have been observed for this eukaryotic microorganism,[5] with a majority of transcripts displaying half-lives in the range of 10–20 min.[7] Although the available literature is less abundant for prokaryotes, shorter half-lives, with mean values of less than 10 min, have been reported for the bacterial models *Escherichia coli*[8,9] and *Bacillus subtilis*.[10] Slightly lower values have been obtained during the exponential growth of *Lactococcus lactis*[11] and *Staphylococcus aureus*.[12] Altogether these global studies not only report average values in the same range of magnitude but also highlight a wide variation of half-lives among mRNAs. Furthermore, global stabilities have been observed to be dependent on growth conditions.[11,12] For example, the average half-life for mRNAs in *L. lactis* increases from 5.8 to 19.4 min when comparing exponential and carbon starvation phases, respectively.[11] It has also been noted that genes which share related biological functions usually display similar messenger decay rates.[8–10] For instance, most house-keeping genes have long half-lives. Proteins which are central in *E. coli* protein–protein interaction network also tend to be encoded by stable transcripts.[13]

The majority of RNA molecules are subjected to regulation and, as is the case for mRNA, their decay can be influenced by growth conditions. Independently of the conditions, the two RNA categories involved in protein synthesis, that is, ribosomal and transfer RNAs, are considered to be more stable than mRNA. Although sRNAs were initially believed to be rather stable RNAs, it has since been shown that they can also be quite susceptible to degradation.[14,15] Plasmid-encoded antisense RNAs have a wide range of half-lives, spanning from less than 2 min to more than 32 min.[16] While studies undertaken to date have mainly focused on the identification of new sRNAs, tools for assessing sRNA differential expression and stability are becoming increasingly available[17] and this will greatly impact the current knowledge of gene expression.

RNA DEGRADATION MECHANISMS

The Original Model and the Initiator RNase E

Turnover of RNA molecules involves cleavage reactions that are carried out by ribonucleases (RNases), a diverse collection of cellular enzymes, whose functions and properties have been elucidated through the study of mutants.[18,19] Although *E. coli* possesses a plethora of RNases only a few are devoted to the RNA degradation. The conventional model for RNA decay in this bacterium usually begins with an endonucleolytic cleavage at one or more internal sites on the RNA molecule (Figure 1(a)). Two endonucleases have been associated with the initial cleavage event: RNase III and RNase E. However, RNase E is believed to be the main endonuclease involved in the RNA turnover in *E. coli*.[18] It is a single-stranded endonuclease that exhibits a preference for A/U-rich regions in close proximity to stem-loops.[20,21] This

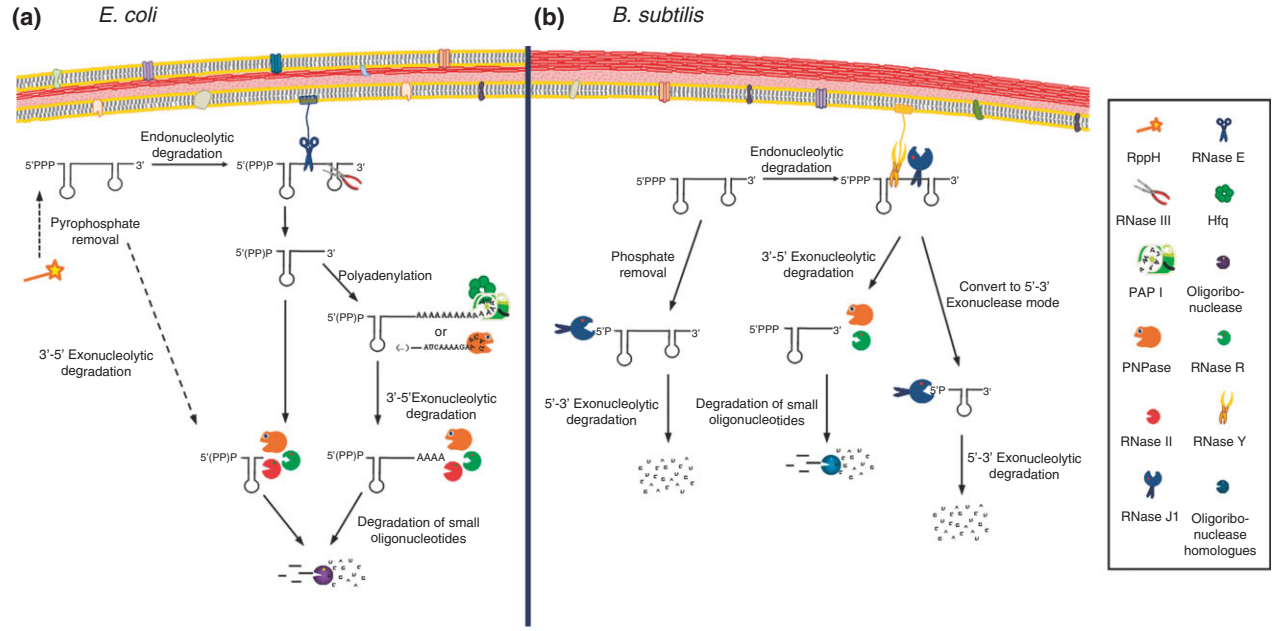

FIGURE 1 | Mechanisms of RNA decay in the Gram-negative and Gram-positive bacterial models. (a) In *E. coli* the decay of the majority of transcripts starts with an endonucleolytic cleavage by RNase E. The enzyme has a preference for 5′-monophosphorylated substrates. A possible pathway for RNase E cleavage involves a primary cleavage by the RNA pyrophosphohydrolase RppH, which converts the 5′-triphosphorylated terminus of primary transcripts to monophosphate. However, some substrates are cleaved by RNase E regardless of the 5′-phosphorylation status, through an alternative pathway called 'bypass' or 'internal entry', which involves the direct entry of RNase E at single-stranded sites. RNase III is double-stranded specific and can also initiate the decay of structured RNAs. After endonucleolytic cleavage, breakdown products are ready for exonucleolytic digestion by any of the three main exonucleases in this bacterium. Unlike RNase R, both RNase II and PNPase are sensitive to secondary structures. Exonucleolytic activity is promoted by the 3′-polyadenylation of substrates. The activity of PAP I, the main polyadenylating enzyme in *E. coli*, is modulated by the RNA-chaperone Hfq. PNPase can synthesize heteropolymeric tails that also facilitate degradation. Cycles of polyadenylation and exonucleolytic degradation have been proposed as one way to overcome secondary structures. A minor alternative pathway in the cell is the direct exonucleolytic degradation of full length transcripts (represented by a dashed arrow). Exonucleolytic degradation releases short fragments which are subsequently degraded to mononucleotides by oligoribonuclease. (b) In *B. subtilis*, transcripts can be degraded from the 5′-end through the 5′–3′ exonuclease activity of RNase J1, or they can be first endonucleolytically cleaved. The 5′–3′ exonuclease activity of RNase J1 is blocked by 5′-PPP, suggesting primary phosphate removal. The endonucleolytic cleavage can be either performed by RNase J1/RNase J2 or RNase Y. The breakdown products can be then further degraded by the 3′–5′ exonucleases, PNPase and RNase R (unprotected 3′ ends), or by the 5′–3′ exonuclease activity of RNase J1 (newly generated monophosphorylated 5′-ends). RNase J1 is able to fully degrade its RNA substrates to mononucleotides. The final products released by RNase R and PNPase are further degraded by the oligoribonuclease homologues in *B. subtilis*. Here we represent ribonucleases acting independently. However some of these enzymes can act together in degradation complexes. For instance in *E. coli* the degradosome (RNase E, PNPase, RhlB and enolase) and in *B. subtilis* the putative complex formed by RNase J1/J2, RNase Y, PNPase, the RNA helicase CshA and two glycolytic enzymes.

characteristic is also shown by its paralogue, RNase G. This endonuclease, which has a strong resemblance with the amino-terminal portion of RNase E,[22] is also involved in the degradation and processing of RNA.[23] Both enzymes display higher activity over substrates bearing a monophosphorylated than over substrates with a triphosphorylated 5′-end.[23] Nonetheless, some substrates are cleaved by RNase E regardless of the 5′-phosphorylation status. This occurs in molecules with multiple single-stranded sites that allow the direct entry of RNase E through a different pathway, called 'bypass' or 'internal entry'.[24,25]

RppH is an RNA pyrophosphohydrolase that removes the pyrophosphate from the 5′-termini and preferentially acts on single-stranded RNA. The discovery of this enzyme presented an alternative pathway in which the initial event is non-nucleolytic.[26] Conversion of 5′-triphosphate to 5′-monophosphate by RppH provides the ideal substrate for RNase E, and the preference of RppH for single-stranded RNA explains why 5′-stem-loops are mediators of stability. Ribosome loading is also known to mediate RNA stability. A poor ribosome binding site, possibly by increasing the distance between the actively translating ribosomes, exposes putative internal cleavage sites and may increase message instability.

The catalytic domain of RNase E lies in the N-terminal region, which is highly conserved

and essential for cell viability.[27] The C-terminus forms a scaffold for interactions with other proteins, which together form the degradosome, the main RNA degradative complex in *E. coli*. The additional presence of a membrane-binding domain in the C-terminal region directs this multiprotein complex to the membrane, which is indicative that RNA degradation is a compartmentalized process.[28] The RNA degradosome can undergo changes in composition depending on the growth or stress conditions.[29,30] For instance, two different RNA helicases are known to associate with RNase E depending on the temperature.[29,31] This remodeling of the degradosome strongly affects its RNA target spectrum.[30] In *E. coli* under normal growth conditions, the major components of the degradosome, in addition to RNase E, are the exonuclease PNPase, the helicase RhlB and the glycolytic enzyme enolase.[31,32] *Pseudomonas syringae*, on the other hand, has selected RNase R as a degradosome component, despite possessing PNPase.[33] This complex of enzymes assures the coordination of the endo- and exonucleolytic degradation of an RNA molecule. After the initial endonucleolytic cleavage step the upstream fragment, lacking the 3′-terminal hairpin, can be readily digested by 3′-exonucleases. The activity of these enzymes is impaired by a 3′-stem-loop, which protects the majority of the primary transcripts.[34] The downstream fragment generated after the initial endonucleolytic cleavage is usually more prone to degradation. It bears a monophosphorylated 5′-end and therefore may be the ideal substrate for an additional cleavage by RNase E. The turnover of *malEF* transcript illustrates how the endo- and exonucleolytic enzymes can act in a concerted way. PNPase degradation of *malEF* is only accomplished in the presence of RNase E and RhlB, indicating that the degradosome participates in its degradation.[35]

Three exonucleases are mainly involved in RNA decay in *E. coli*: PNPase, RNase R and RNase II (Figure 1(a)). All of these enzymes degrade RNA processively and nonspecifically from the 3′-end. While PNPase is a phosphorolytic exonuclease yielding nucleoside diphosphates as reaction products, both RNase R and RNase II catalyze the hydrolysis of the RNA substrates, producing nucleoside monophosphates. Among the three, only RNase R is able to digest structured RNA by itself.[34] The degrading activity of PNPase or RNase II is stalled by the presence of secondary structures.[36] However, PNPase can also proceed through extensive folded RNA when acting in association with other proteins. Its association with the helicase RhlB or integration into the degradosome allows the unwinding of the RNA stem-loops.[37] Surprisingly, the PNPase homologue of *Thermus termophilus*, whose optimal temperature is 65°C, has been reported to completely degrade RNAs with stable intramolecular secondary structures without the aid of a helicase.[38] Nonetheless, both PNPase and RNase R require a minimal 3′-overhang of 7–10 unpaired nucleotides in order to be able to bind and initiate digestion of an RNA molecule.[39] By providing a single-stranded platform for the initiation of the exonucleolytic attack, the degradation of RNA molecules containing 3′-stem-loops is stimulated by the addition of poly(A) tails to the 3′-end of the RNA molecules (for details see '*Polyadenylation*' section). These poly(A) tails constitute the preferred substrate for PNPase and RNase II.[40,41]

None of the three 3′-exonucleases seems to be indispensable for *E. coli* growth at optimal temperature. However the combined absence of both PNPase and RNase II or PNPase and RNase R is lethal for the cell, indicating some overlapping role between these exonucleases.[42] For instance, both RNase R and PNPase are involved in the degradation of rRNA fragments, whose accumulation was proposed to lead to cell death.[42] A transcriptome analysis revealed that, although RNase II accounts for 90% of exonuclease activity in the cell, PNPase probably plays a greater role in mRNA degradation than previously thought.[43,44] RNase II is the major exonuclease involved in *E. coli* RNA decay and other enterobacteriaceae but, this enzyme is absent in several other bacterial species, such as *B. subtilis*, *Legionella pneumophila* and *Streptococcus pneumonia*e, in which RNase R is the only hydrolytic 3′–5′ exonuclease.[45,46] In *B. subtilis* the RNA decay is primarily phosphorolytic and this major activity is attributed to PNPase.[18] Regarding the main exonucleases, PNPase is the only one found in *Streptomyces*, thus constituting an essential protein in these organisms.[47] Conversely, the unique exonuclease in *Mycoplasma genitalium* is RNase R, which is thus essential.[48]

The degradative action of the ribonucleases described above releases RNA fragments of 2–5 nucleotides, whose accumulation may be deleterious to the cell.[49] *E. coli* possesses another exonuclease, termed oligoribonuclease, which acts as a scavenger of these short oligoribonucleotides[50] (see Figure 1(a)). This essential enzyme processively hydrolyses RNA in the 3′–5′ direction. Overall, oligoribonuclease is a finishing enzyme in RNA metabolism, and the presence of proteins with analogous functions seems to be widespread. Two homologues, NrnA and NrnB, have been described in *B. subtilis*.[51]

The Case of the Double-Stranded Specific RNase III

Even though RNase E has been considered the main enzyme in *E. coli* that catalyzes the initial cleavage event, the RNase III family of enzymes has emerged as an important group of endonucleases in the control of RNA stability.[52] RNase III deletion in *E. coli* causes a slow growth phenotype,[53] while its homologue in *B. subtilis* is essential for viability.[54] A second *B. subtilis* RNase III-like enzyme (called Mini-III) has recently been described.[55] Both enzymes seem to act mostly in bacteriophage mRNA and rRNA processing, since no endogenous mRNA targets are known.[56]

RNase III is specific for double-stranded RNA and its role in RNA turnover has been associated with the removal of protective stem-loop structures that act as degradation barriers[18] (Figure 1(a)). Additionally, RNase III has recently been implicated in the decay of sRNA/mRNA complexes, which constitute an optimal substrate for this enzyme, upon translational silencing.[15,57] This phenomenon closely resembles siRNA–direct RNA cleavage in eukaryotes, a process that involves enzymes of the RNase III family.

An Alternative Mechanism Involving Different Endonucleases

Despite its essential role in *E. coli* RNA turnover, RNase E homologues are absent in numerous bacterial species. This is the case of the model organism *B. subtilis* and is a common characteristic of the low G/C content Gram-positive bacteria. For quite some time, a good candidate that could have the analogous role of RNase E was not found, leaving no clue for the process of *B. subtilis* RNA degradation. One answer came from the discovery of two different ribonucleases, RNase J1 and J2, which are present in almost all bacteria lacking RNase E.[58] Curiously, both RNase E and RNase J1 orthologs have been found in *Sinorhizobium meliloti*.[59] Although there is no sequence homology, the RNase J enzymes share a similar architecture with RNase E[60] and exhibit equivalent endonucleolytic activity. RNase J1, which is essential for cell viability, is involved in RNA turnover[61] (Figure 1(b)). Surprisingly, this enzyme is also able to catalyze the exonucleolytic degradation of RNA in the 5′–3′ direction.[62] To date this is the only 5′-exonuclease known in prokaryotes and its discovery has had important implications in the RNA decay model. The exonucleolytic decay from the 5′-end may explain the stabilizing effect conferred by 5′-stem-loops, 5′-protein binding and 5′-ribosome stalling in *B. subtilis*.[56] It has been suggested that this dual-function enzyme (alone or in complex with RNase J2) catalyzes not only the endonucleolytic cleavage of an RNA substrate but also continues the degradation of the generated 5′-end by switching to the 5′-exonucleolytic mode.[60] Interestingly, only the exonucleolytic activity of RNase J1 is dependent on the 5′-end phosphorylation status, as it is blocked by triphosphorylated RNA.[60] The complex formed by RNase J1 and J2 changes their individual cleavage activities and specificities.[63]

Another insight into the RNA degradation mechanism of *B. subtilis* was the recent discovery of RNase Y, an essential single-stranded endonuclease (Figure 1(b)), whose deletion was reported to increase the bulk mRNA half-life in this bacterium.[64] Like RNase E and RNase J1, RNase Y is sensitive to the phosphorylation state of the 5′-end, exhibiting a marked preference for monophosphorylated RNA. Hence, two essential enzymes in *B. subtilis* RNA decay are dependent on a monophosphorylated 5′-end. Indeed, there are indications of the existence of an RppH-like enzyme in *B. subtilis* (Belasco and Condon, personal communication).

The presence of a putative N-terminal transmembrane domain in RNase Y suggests membrane localization further extending the analogy to RNase E[64] (Figure 1(b)). Evidence for the presence of a complex involving RNase Y, RNase J1/J2, PNPase, the RNA helicase CshA and two glycolytic enzymes has recently been reported.[65] This complex brings together some of the degrading activities necessary to achieve full degradation of an RNA molecule. The RNA fragments released by the RNase Y endonucleolytic cleavage could be good substrates for the 3′-exonucleolytic activity of PNPase and for the 5′-exonucleolytic degradation by RNase J1/J2.[56] This putative degradosome-like complex indicates that the presence of such an RNA degradative machine may be a common feature in prokaryotes, even those that lack an RNase E homologue.

ROLE OF RNA DEGRADATION IN QUALITY CONTROL

Gene mutation, DNA damage, or transcriptional errors may generate damaged mRNAs that are unsuitable for protein synthesis. Additionally, translational frameshifting can also lead to aberrant proteins, whose accumulation may be detrimental to the cell. Hence, bacteria have evolved efficient quality control mechanisms engaged in the rapid degradation of these abnormal transcripts and proteins.

One of the common errors is the presence of a premature stop codon in some messages, which produces a truncated protein. In *E. coli* the fast

degradation of messages carrying a premature stop codon is thought to begin with a 5′-independent RNase E cleavage at internal sites exposed by the premature release of ribosomes.[24] The resulting RNA molecules are further degraded through the pathways described above.

Messages without an in-frame stop codon may lead to ribosome stalling at the 3′-end, significantly affecting translational efficiency. An elegant surveillance pathway, termed *trans*-translation, targets deficient proteins and mRNA for degradation while rescuing stalled ribosomes (see Refs 66 and 67 for a review). This process relies on the association of two molecules: a sRNA called transfer-messenger RNA (tmRNA), and a small RNA-binding protein (SmpB), whose homologues have been identified in every sequenced eubacterial genomes. Some situations that delay the progress of ribosomes during translation were also observed to elicit tagging by the *trans*-translation machinery in a process that involves RNase II.[66,68]

The rapid removal of the defective messages that lead to ribosome stalling is of utmost importance in the prevention of future stalling events. RNase R is associated with tmRNA and SmpB and is involved in the tmRNA-mediated decay[69] as well as in the processing of tmRNA.[70] The mechanism that influences the loading of RNase R onto the defective mRNAs was recently highlighted, whereby RNase R was shown to be recruited through interactions mediated by its C-terminal lysine-rich domain in an SmpB-tmRNA-dependent manner.[71] Indeed, this domain was previously suggested as being involved in interactions with SmpB.[72]

Finally, errors in macromolecular processes like tRNA and rRNA synthesis also occur. Li et al. had shown that an aberrant precursor tRNA molecule is degraded by a mechanism that involves poly(A) polymerase and PNPase,[73] leading to the proposition that polyadenylation could serve as a signal to promote degradation of defective tRNAs. PNPase and RNase R have been implicated in the removal of defective rRNAs.[42]

STRUCTURAL DETERMINANTS OF THE RNA DEGRADING ENZYMES

The intrinsic degradative nature of ribonucleases and the fact that they share the cellular environment with a pool of different types of RNA molecules raises some questions: How do these 'molecular killers' specifically select their targets? What are the structural determinants that allow these enzymes to spare some molecules while dictating the destruction of others? As underlined above there are several features of the RNA molecules that are key factors in determining their fate. The interplay between the structural determinants of the enzymes and their specific degradation preferences, depending on the characteristics of each RNA molecule, seems to dictate the final decision.

RNase E, the main endonuclease of numerous species including *E. coli*, organizes its catalytic domain, as a dimer of dimers in a final homotetramer quaternary structure[74] (Figure 2). Each protomer contains the following structural domains: S1, RNase H, DNase I and a small domain that is responsible for dimer–dimer interaction. The arrangement of the domains within each dimer resembles the blades and handles of an open pair of scissors. The crystal structure explains some features of the enzyme and suggests a mechanism for RNA recognition and cleavage.[75] The influence of 5′-phosphorylation is a consequence of the pocket formed between the S1 and the RNase H subdomains, which binds 5′-monophosphorylated RNA and promotes downstream degradation. After binding, a conformational change induced by the movement of the RNA-binding domains clamps the substrate down and organizes the active site.[75] The catalytic site contains conserved residues of the DNase I domain and a single metal-binding site that coordinates an Mg^{2+} ion implicated in catalysis. The internal flexibility within the quaternary structure may be related to the deformation required to accommodate structured RNA for processing by internal entry. An amphipathic segment at the C-terminal region of RNase E directs the enzyme to the inner membrane.[76] Membrane localization could be determinant for spatial discrimination of the RNA substrate.

E. coli RNase III is the prototype of the RNase III family of enzymes, which includes eukaryotic enzymes such as Dicer and Drosha. The bacterial enzymes are the simplest, containing an N-terminal endonuclease domain (NucD) characterized by a 9-residue consensus sequence known as the RNase III signature motif, and a double-stranded RNA binding domain (dsRBD) in the C-terminus. The enzyme is active in the homodimeric form and the crystal structure of the *Aquifex aeolicus* RNase III endonuclease domain shows that dimerization creates a large valley that accommodates dsRNA[77] (Figure 2). The catalytic centers are found in the dimer interface, one at each end of the valley. Although dsRNA binding is governed by the combined dsRBD of the dimer, the NucD domains contribute to substrate specificity.[77] Substrate selection consists of a combination of structural and sequence elements, such as the strength of base pairing, the occurrence of

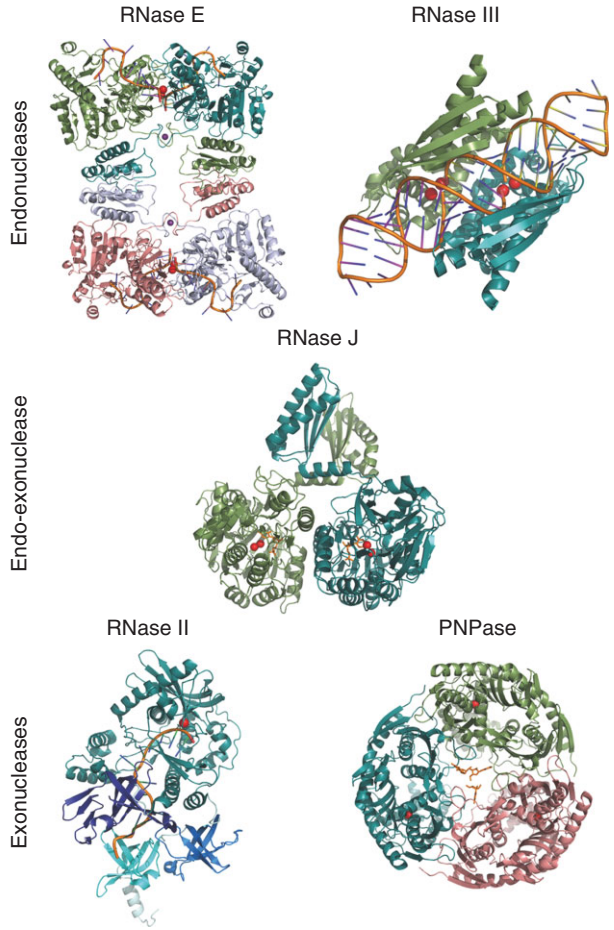

FIGURE 2 | Structures of RNA degrading enzymes in complex with RNA substrates. On the top of the image are shown the crystal structures of endonucleases (catalytic domain of *E. coli* RNase E, PDB ID 2C4R, on the left; *A. aeolicus* RNase III, PDB ID 2NUF, on the right). The crystal structures of exonucleases are on the bottom (*E. coli* RNase II, PBD ID 2IX1, on the left; *E. coli* PNPase, PDB ID 3GCM, on the right). The crystal structure of *Thermus thermophilus* RNase J (PDB ID 3BK2), which has a dual function as endo- and exonuclease, is in the middle. RNA substrates in complex with the enzymes are colored in orange and the metal ions that assist catalysis are shown as red spheres. Purple spheres denote the Zn^{2+} ions important for maintenance of the principal dimers in the RNase E quarternary structure. Otherwise the colors are unrelated to the functional domains of the enzymes, but represent different protomers in the quaternary structures except in RNase II, which is active as a monomer. In this case, each color identifies a different domain (CSD1 and CSD2 are shown in cyan and light blue, respectively; S1 is shown in dark blue and the catalytic domain RDB is colored in dark cyan). A model for RNase R has been proposed but the structure is not yet available. However functional and structural data available indicates that the structure will be quite similar to RNase II. Structures were drawn using PyMOL (http://pymol.sourceforge.net).

specific nucleotide pairs and the helix length.[78] Several conserved residues in the catalytic centers were shown to be essential for catalysis, which involves amino acids from both subunits, and thus, dimerization of the NucD domains is necessary for RNase III function (see Ref 77 and references therein). The crystal structure indicates that a single RNA cleavage event occurs on each strand of the dsRNA within each cleavage site, generating products with a two-base 3′-overhang. Mg^{2+} is required for the formation of a catalytical competent protein–RNA complex and two divalent cations are coordinated by each active site. When Mg^{2+} is absent the RNA is bound outside the catalytic valley.[79] Indeed, RNase III is also known to be involved in the control of gene expression by binding a dsRNA molecule without cleaving. In this noncatalytic functional form the enzyme plays the role of a dsRNA-binding protein.[80]

RNase J is unique among bacterial RNases in that it possesses both endo- and 5′–3′ exonucleolytic activity.[60,81] Recent structural data shed some light into the dual activity of *Thermus thermophilus* RNase J[60] (Figure 2). The enzyme is active as a dimer in solution, and each monomer contains three distinct domains: a metallo-β-lactamase core, a β-CASP and a C-terminal domain. This last domain may be involved in substrate recognition and maintenance of the dimeric state. Two Zn^{2+} ions, essential for catalysis, are coordinated by residues located deep in the cleft between the β-lactamase core and the β-CASP domain. Specific attack of 5′-monophosphorylated transcripts is related to a binding pocket near the catalytic center that precludes the accommodation of a 5′-triphosphorylated substrate. Only one catalytic center was obvious from the structure, suggesting that a single active site is responsible for the dual activity of RNase J. The distance between the 5′-monophosphate binding pocket and the catalytic center may explain the switching from endo- to exonucleolytic mode. Indeed, the 5′-monophosphate generated after the endonucleolytic cleavage may be directly placed in the binding pocket with a single translocation of the RNA molecule, allowing RNase J to start exonucleolytic degradation.

E. coli RNase II is the prototype of the RNase II family of enzymes, a widespread family that also includes RNase R and the catalytic subunit of the eukaryotic exosome, Rrp44/Dis3. The overall crystallographic structure of *E. coli* RNase II reveals a monomeric protein with four domains: three RNA binding domains comprising CSD1 and CSD2 in the N-terminal region, and an S1 fold at the C-terminus; one RNB catalytic domain in the central region, which is a hallmark of the RNase II family of proteins[82–84] (Figure 2). The RNA binding domains are grouped together in one side of the structure, and play a role in the RNA substrate selection and binding.[83,85]

Interestingly, a truncated enzyme lacking all the RNA binding domains is still able to degrade RNA.[85,86] The structure of the enzyme in complex with an RNA molecule reveals two main noncontiguous interaction points between the protein and the RNA fragment. A 10-nucleotide segment is the shortest RNA able to retain both contacts, explaining why RNase II becomes distributive on substrates shorter than 10 nucleotides.[83] A single amino acid change in the catalytic region alters the final end-product from 4 to 10 nucleotides, probably due to loosening of the RNA substrate at the catalytic site.[87] The access to the catalytic pocket is restricted to single-stranded RNAs by steric hindrance, explaining the inability of RNase II to degrade dsRNA. A specific interaction with ribose rings precludes DNA cleavage.[83] Several residues in the catalytic region are important for catalysis;[83,87–89] however, only Asp209, which is involved in the coordination of Mg^{2+}, is essential.[87] Interestingly, substitution of one conserved glutamine gives rise to an enzyme with highly increased RNA-binding and exonucleolytic activity (120-times higher), consequently called 'super-enzyme'.[89]

RNase R shares a similar domain organization with RNase II, and a three-dimensional model of this enzyme has been proposed based on the structure of RNase II.[87] Besides the domains identified in RNase II, RNase R also contains a helix-turn-helix in the N-terminus,[46] and a highly basic region after the S1 fold at the C-terminus.[86] The RNB domain of RNase R alone is sufficient to bind and degrade an RNA duplex.[85,86] Paradoxically, when the RNA-binding domains are present a short 3′-overhang is necessary in order to initiate degradation. These domains are essential for binding and recruitment of 3′-tailed RNA molecules. The two CSD domains appear to play a role on the recognition of the substrates for degradation, whereas the S1 domain is most likely required to position substrates for efficient catalysis.[85,86] The RNA-binding regions (S1, CSD1, and most importantly CSD2) have been suggested to possess intrinsic helicase activity,[90] a hypothesis that still needs further experimental support. Mutation analysis in the nuclease domain identified important residues for the nuclease activity and for substrate binding, which may contribute to the ability of RNase R to degrade structured RNAs.[85,91] Nevertheless, only the resolution of its three-dimensional structure will allow full understanding of its remarkable mode of action.

PNPase belongs to the PDX family of exonucleases, which also includes the core of the exosome in archaea and eukaryotes.[92] The crystal structures of *Streptomyces antibioticus* and *E. coli* PNPase reveal a homotrimeric subunit organization with a ring-like architecture[93–95] (Figure 2). This structure closely resembles the doughnut-like shape of the archaeal and the eukaryotic exosomes.[92] Each monomer comprises two RNase PH domains (PH1 and PH2) forming the catalytic site (PNPase core), which are connected by an α-helical domain. Several mutations introduced into this core have been shown to influence phosphorolytic and polymerase activities of the enzyme.[96,97] In addition, two RNA-binding domains S1 and KH have been found in the C-terminus. These domains are required not only for proper binding, but also contribute to the formation of a more stable trimeric structure.[95,98,99] In the quaternary structure, the RNA-binding domains are grouped in one face of the trimer, while the active site is located in the opposite side. The association of the three subunits encloses a central channel through which the RNA molecule travels in the direction of the active site. A properly constricted channel and the conserved basic residues in the neck region play critical roles in trapping RNA for processive degradation. RNA translocation is dynamic, and PNPase undergoes conformational changes at the central channel and its neighboring regions, while directing the RNA to the active center.[93–95] Mg^{2+} is required for PNPase enzymatic activity, though Mn^{2+} can also support catalysis. The metabolite citrate has recently been shown to directly modulate the enzyme's activity, connecting RNA degradative pathways with the central metabolism.[100]

In this section, we have highlighted the structural features of the ribonucleases that are specifically involved in the recognition of a given RNA molecule. In light of the enzymes' spatial architecture, it was mentioned how the sequence and structural elements within the transcript regulate both its rate of decay and the primary nucleases involved. Furthermore, it was shown that some ribonucleases can also be influenced by the spatial compartmentalization within the cell, which may modulate their access to different RNA molecules.

OTHER PLAYERS

Polyadenylation

Polyadenylation is a post-transcriptional event that involves the addition of untemplated adenosine residues to the 3′-ends of RNA substrates. This widespread phenomenon occurs in bacteria, organelles, archaea, and in the nucleus and cytoplasm of eukaryotic cells. Eukaryotic poly(A) tails, present in the majority of mRNAs, are usually long, uniform in length and have traditionally been viewed as a stabilizing element. However, recent studies have also

shown evidence of a polyadenylation-induced decay of nonfunctional RNAs in eukaryotes.[101] Prokaryotic polyadenylated transcripts are generally low in abundance and poly(A) tails are very short and unstable. This fact, together with a lack of evidence for a physiological role, made polyadenylation in bacteria almost overlooked for several years after its discovery. It has been estimated that less than 2% of total RNA in *E. coli* is polyadenylated. Despite this intriguingly low percentage, a transcriptome comparison between the wild-type and a strain defective in polyadenylation has indicated that the majority of transcripts (~90%) undergo some degree of polyadenylation during exponential growth, either as full-length transcripts or decay intermediates.[102] The rapid turnover of polyadenylated mRNAs accounts in part for their low abundance.

Poly(A) tails provide a single-stranded extension region that works like a 'toe-hold' upon which exonucleases can bind and initiate decay[103] (Figure 1(a)). Polyadenylation therefore facilitates exonucleolytic activity. Paradoxically, the higher affinity of RNase II for these poly(A) stretches has been shown to protect mRNAs from degradation, as this enzyme degrades the tails that allow PNPase and RNase R to proceed through secondary structures.[41,43]

Initially identified almost 50 years ago in *E. coli*,[104] Poly(A) Polymerase I (PAP I) is responsible for ~90% of the polyadenylating activity in the cell.[105] The enzyme catalyzes the addition of homopolymeric poly(A) tails (15–30 nt long in average) to 3′-hydroxyl termini of RNA molecules using ATP as a substrate.[102,106,107] However, deletion of its cognate gene (*pcnB*) has only a moderate effect on growth[108] and does not abolish all the polyadenylating activity in the cell.[109] PNPase can also synthesize A-rich heteropolymeric extensions through its reverse activity (synthetic instead of degrading),[109] although to a much lesser extent. This PNPase activity has recently been shown to change in response to adjustments of cyclic-di-GMP levels in an O_2-dependent way.[110] In many prokaryotic organisms, archaea, and organelles of prokaryotic origin lacking a PAP I protein, polyadenylation has been shown to be carried out by PNPase.[111] It has been speculated that the evolutionary precursor of PNPase was the first enzyme to produce these tails. Much later, PAP I would have been acquired by bacteria such as *E. coli*. The enzyme, already specific for ATP (the 'energy currency' of the cell), produced the dominant homopolymeric poly(A) tails. *B. subtilis,* in which as much as 15–25% of total RNA was estimated to be polyadenylated,[112] lacks an identifiable PAP I homologue. Nonetheless, similar polyadenylated and heteropolymeric ends have been observed at the 3′-ends of RNA isolated from wild-type and PNPase mutant strains, indicating that PNPase is not the only enzyme responsible for the addition of nucleotides to the 3′-end of RNAs in this organism.[113]

Regarding the nature of the poly(A) tail, several observations indicate that transcripts which terminate in a Rho-dependent fashion tend to contain only heteropolymeric tails generated by PNPase. On the other hand, Rho-independent transcription terminators serve as polyadenylation signals for PAP I.[102,106] Moreover, heteropolymeric tails are mainly added to breakdown products, whereas poly(A) tails are added to both breakdown and full-length transcripts.[105] In stationary phase, the addition of heteropolymeric tails is predominant over the homopolymeric tails commonly found in exponentially growing cells.[114] The lack of energy resources in stationary phase could be a reason for this choice, since the generation of adenylated tails at the expense of several ATP molecules is more justifiable under exponential growth.[114] Moreover, polyadenylation promotes high mRNA turnover rates which are characteristic of the exponential phase.[109]

In both bacteria and organelles, RNA breakdown products generated by endonucleolytic cleavages are considered the most favored substrates for 3′-tailing.[115–118] Single-stranded segments at either 5′- or 3′-end of RNA molecules and monophosphorylation at an unpaired 5′-terminus were reported to increase the RNA susceptibility to polyadenylation by PAP I.[119] This suggests that the endonucleolytically-generated RNA fragments containing single-stranded monophosphorylated 5′-termini would be preferential substrates for 3′-end polyadenylation. However, this is not always required as transient poly(A) tails have been found at the native 3′-ends of RNA molecules.[103] In *E. coli,* the low level of PAP I (32–50 molecules per cell) is also likely to be a limiting factor in substrate selection.[102,106] PAP I over-synthesis is highly toxic, which may be the reason for such a low enzyme level.[108,120]

It has long been presumed that 3′-polyadenylation was only restricted to mRNAs, nevertheless PAP I and PNPase can polyadenylate almost any RNA species, including rRNAs, tRNAs, and sRNAs. Interestingly, while the tails found on rRNAs resemble the ones found on mRNAs, the tails on tRNAs and sRNAs tend to be very short (1–8 nt).[121,122] A few sRNAs have been reported to be destabilized by polyadenylation.[122–126] Polyadenylation was also implicated in the promotion of the exonucleolytic degradation of defective tRNAs.[73] A defective $tRNA^{TRP}$ does not accumulate to the normal wild-type

levels due to the rapid degradation of its precursor. Using PAP I and/or PNPase mutant strains, it has been proposed that polyadenylation of the defective precursor serves as a signal to promote its fast degradation by PNPase.[73]

PAP I has been reported to have physical interactions with Hfq, PNPase, RNase E, and the RNA helicase RhlB,[106,127] which suggests that the polymerase could act as part of a multiprotein complex.[106] It has been demonstrated in *E. coli* that PAP I is localized either in the membrane or in cytosol, depending on the growth phase.[128,129] Such localization may, however, be indirect through a loose association with RNase E.[28,76] The release of PAP I from the membrane in the transition from the exponential to the stationary phase has been found to be dependent on the adaptor protein SprE (RssB), previously known for its role in governing the stability of the alternate σ factor RpoS.[128] SprE has also been reported to be required to maintain the association of PAP I and Hfq with the degradosome during stationary phase.[130] In addition, microarray data have revealed that polyadenylation and turnover of specific *E. coli* transcripts can be modulated by SprE.[128]

Hfq

Hfq is an RNA chaperone known to be involved in the stabilization and/or degradation of many RNAs. This widespread and highly abundant post-transcriptional regulator belongs to the Sm/Lsm family of RNA binding proteins. The active form of Hfq has a doughnut-shape homohexameric ring structure that displays the highest affinity for short single-stranded stretches of adenines and uridines adjacent to stem-loop structures.[131] The protein has at least two RNA-binding sites located in the conserved N-terminal portion: the proximal site, which binds sRNAs and mRNAs; and the distal site, specific for poly(A) stretches.[132] This protein can directly interact with PAP I and/or change its activity from distributive to processive[106,133] (Figure 1(a)). Actually, Hfq targets many mRNAs for degradation by binding to their poly(A) tails and stimulating polyadenylation.[133,134] Paradoxically, Hfq stabilizes sRNAs, probably because its binding protects them from ribonuclease attack as there is an overlapping of recognition sites between Hfq and RNase E.[135] Hfq has also been shown to interact with PNPase and RNase E.[105,106,136] The formation of variable ribonucleoprotein complexes between RNase E and Hfq/sRNAs specifically destabilizes the mRNA target.[136]

The pleiotropic phenotype of *hfq* null mutants reveals that this protein acts on several pathways of *E. coli* metabolism.[137,138] Indeed, Hfq is currently recognized as a key factor in regulation by sRNAs. This protein promotes sRNAs interaction with their mRNA targets (see details in the next subsection),[15,139] although the precise mechanism by which Hfq brings mRNAs and sRNAs together is not completely understood. The Hfq binding may unfold or weaken RNA secondary structures, allowing sRNAs to access their targets.[140,141] Alternatively, the simultaneous interaction between the sRNA with the Hfq proximal site, and the mRNA with its distal site, may raise the local concentrations of the two RNAs, thereby increasing the probability of sRNA–mRNA interaction.[142] When the interaction between the two RNA molecules is stable, Hfq dissociates or is proteolytically removed from the complex.[141]

sRNAs

sRNAs have been extensively studied over the last years because of their high importance in the post-transcriptional regulation of bacterial gene expression. They have become another important factor to consider in the global picture of RNA turnover. Their action can directly trigger the degradation of specific mRNAs, thus changing gene expression.

The mechanisms of regulation by sRNAs are very complex and reports of different modes of action are becoming increasingly common in the literature. Some interact with a protein to modify its activity. For instance, CsrB and CsrC tightly regulate the activity of the global post-transcriptional regulator CsrA by binding and sequestering several of these proteins simultaneously.[143] The vast majority of the small RNAs belongs to the class of antisense sRNAs that act by base-pairing with their mRNA targets. These sRNAs usually bind their mRNA targets to repress translation and/or accelerate their degradation. Binding of the sRNA usually sequesters the ribosome binding site (RBS) and, consequently, prevents the 30S ribosome loading. Following translational repression, the mRNA target often becomes substrate for RNase E or RNase III.[144] The action of sRNAs is, however, not limited to the RBS. Recognition of the target upstream of its 5′-UTR[145–147] and binding inside the coding sequence of the mRNA target[148] has also been reported. Although less numerous, examples of gene expression activation by sRNAs are also known. For instance, the translation of the *rpoS* mRNA is positively regulated by the action of the sRNAs DsrA, RprA, and ArcZ.[149–151]

Antisense sRNAs can act either in *cis* or in *trans* depending on their genomic location in relation to their mRNA target(s). MicA sRNA is the only known case of a sRNA in *E. coli* which targets both in *cis* and in *trans*.[152] *Cis*-encoded sRNAs are transcribed from the same *locus*, but in the opposite sense. The best

known examples are those involved in the replication of plasmids.[153] Nevertheless, the bulk of antisense sRNAs are *trans*-encoded, being transcribed from a distinct *locus*, and these are normally induced under stress conditions. One-third of these sRNAs repress the synthesis of outer membrane-proteins.[154] As the complementarity of *trans*-encoded sRNAs with the respective targets is imperfect they typically require Hfq for target interaction and/or intracellular stability. Hfq-dependent antisense regulation is widespread in Gram-negative bacteria but only one example was reported in the Gram-positive bacteria *Listeria monocytogenes*.[155]

REGULATION OF RNases

As effectors that rapidly modulate the levels of RNAs, the expression of RNases must be tightly regulated in a process that responds to many different signals and which may relevantly affect RNA turnover rates. This regulation is known to occur in several ways. RNases can regulate their own levels (autoregulation) and/or be regulated by other ribonucleases (cross-regulation). Additionally, their intracellular bulk level can be affected by the medium composition and other environmental factors that alter the cell growth rate. These and other approaches of regulation of the degradative ribonucleolytic machinery in prokaryotes are discussed below in further detail.

Autoregulation and Cross-Regulation

Both RNase E[156] and RNase III[157] have the ability to control the decay of their own mRNAs, thereby regulating their own expression and maintaining the enzyme levels within a narrow range. In both cases, a cleavage that occurs in the 5′-UTR region of the mRNA promotes their decay. PNPase is able to regulate its own expression as well, but in an RNase III-dependent manner.[158] PNPase acts in concert with RNase III to degrade a double-stranded region in the 5′-end of *pnp* mRNA. The removal of this region impairs translation and allows further degradation of *pnp* transcript by RNase E.[159] PNPase expression is also regulated by RNase II.[160] This latter case constitutes an interesting example of cross-regulation: in the absence of RNase II, PNPase levels are increased, while PNPase overexpression leads to a decrease in RNase II activity. PNPase controls RNase II activity by degrading its mRNA.[161] RNase III and RNase E endonucleases are also involved in the control of RNase II expression.[162] While RNase III regulates RNase II by affecting PNPase levels, RNase E directly intervenes in the degradation of *rnb* mRNA. RNase E is also the main enzyme responsible for the processing of *rnr* transcripts encoding RNase R.[163]

Regulation by Environmental Conditions and Other Cellular Modulators

RNase E is responsible for many processes of RNA decay and maturation. Therefore, the enzyme must be tightly regulated because any change in the level of its expression has important cellular repercussions. As discussed above, the 5′-end phosphorylation state, folding and translation of a given mRNA substrate can modulate the RNase E cleavage efficiency by altering the enzyme's accessibility to the transcript. In addition, RraA and RraB (regulators of RNase activity A and B, respectively), interact with RNase E to inhibit its activity.[30,164] In addition to these factors, environmental conditions such as temperature and medium composition have been reported to directly affect the activity and cellular concentration of this endonuclease.[165–167] As a consequence, the RNA processing and stability of specific transcripts is affected.[165–167]

RNase R is a ribonuclease whose levels also change in response to different environmental stimuli.[168] These include the entry into stationary phase (2-fold), heat-shock (~2-fold), and cold-shock (7–8 fold).[70,168,169] From the different stress conditions analyzed, cold-shock treatment results by far in the highest up-regulation effect over RNase R. This marked increase in RNase R levels is probably related to its ability to overcome RNA secondary structures, whose formation is thermodynamically favored under low temperatures. Northern blot analysis of the transcript's decay has indicated an increase in *rnr* stability during cold-shock.[70] Moreover, Western blot analysis of RNase R degradation after translational arrest has revealed that the protein itself is highly stabilized under cold-shock, stationary-phase and growth in minimal medium.[72] It has been shown that the tmRNA–SmpB system can be responsible for the low stability of the RNase R protein in exponential-phase cells, through interaction with the region encompassing the S1 domain and the C-terminus of the exonuclease.[72] Albeit to a lower level, growth at low temperatures also induces PNPase expression, which is an essential enzyme in this condition.[170,171] Moreover, certain mutations of the PNPase RNA binding domains have been shown to confer a cold-sensitive phenotype.[97,98,172]

Like RNase R, RNase II stability is post-translationally regulated. Deletion of *gmr* (gene modulating RNase II), a gene that lies just downstream of *rnb*, causes the accumulation of this exonuclease by increasing the protein stability more than 2-fold.[173]

RNase II is also more abundant in rich medium compared to minimal medium and is sensitive to the nitrogen content of the medium. This regulation is abolished in a *gmr* mutant.[173] One possible explanation is that the PAS domain of Gmr protein can act as a sensor, which monitors nutrients in the growth medium and carries the signals to the proteolytic enzymes responsible for RNase II degradation. Interestingly, beyond being regulated post-transcriptionally (at the level of mRNA and protein), RNase II has two promoters and its expression is also controlled at the transcriptional level.[160,174,175]

Polyadenylation is implicated in the destabilization of a variety of transcripts but it also arises as a factor controlling RNases levels in the cell. A high intracellular level of poly(A) tails stabilizes *pnp* and *rne* transcripts, thereby leading to increased PNPase and RNase E levels, respectively.[176]

The activity of RNases represents a checkpoint on RNA regulation. Accordingly, their expression has to be tightly regulated in different ways, as small variations on their levels may have a tremendous impact on global RNA decay.

CONCLUSIONS

RNA degradation is an intricate mechanism and a process that plays a fundamental role in the regulation of gene expression. The steady-state level of a given transcript is dependent on a high level of coordination between the different players involved in transcription and degradation. This complex network permits a rapid response to challenging conditions, and it is therefore not surprising that RNA degradation is not exclusively deterministic but is also controlled by external stimuli. RNA decay is a major link in the chain of bacterial adaptation, a key for survival and development.

In this article, we aimed at providing an up-to-date picture of RNA stability/decay and its control in prokaryotes. It involves numerous players and relies on several features. However, the main effectors are ribonucleases, whose diversity, structures, targets, and modes of action can vary significantly, providing multiple solutions for a similar issue. Ribonucleases can act independently, or in a concerted way, as well as in higher order protein complexes such as the degradosome. Their activity can be modulated by regulatory proteins. Besides this regulatory network, the fate of an RNA molecule is intimately related to its sequence and structural features. Indeed, the characteristics of an mRNA molecule can determine ribosomal pausing, targeting by sRNAs, and also elect which RNases will be able to act.

Novel players that influence RNA stability are still being discovered. These new findings not only increase our knowledge of this topic but often challenge the conventional models. For instance, the finding of the RNA pyrophosphohydrolase RppH in *E. coli* revealed an alternative pathway for degradation. The homologue in *B. subtilis* was just recently proposed (Belasco and Condon, personal communication). The ability of the RNase J enzymes to cleave RNA in the 5′–3′ direction has also restructured the conventional view of RNA decay in prokaryotes. Another example is the discovery of sRNAs as essential regulators of mRNA stability, which has significantly altered the established models of RNA turnover. Degradation of these small molecules is in turn directed by RNases and other factors, namely polyadenylation. Their degradation can alter the concentration of the target mRNA by directly modifying the cellular concentration of the sRNA.

There are also indications of the existence of a cellular compartmentalization in bacteria, which could help to synchronize regulatory events. Certain mRNAs have been shown to migrate to particular cellular domains where their future protein products are required.[177] In another study the mRNAs studied exhibited limited dispersion from their site of transcription. In this latter case the chromosome was proposed to be a spatial organizer of mRNA and related processes.[178] Different mRNAs were studied and this may account for these apparently divergent observations. The identification of additional players and regulatory processes is essential for fully understanding RNA decay. Although there has been tremendous progress in our understanding of the post-transcriptional control of RNA stability and decay, the continuing discovery of new processes illustrates that the intricacies of RNA degradation are still far from being completely understood, and a remarkable amount remains to be learned.

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REFERENCES

1. Russell JB. The energy spilling reactions of bacteria and other organisms. *J Mol Microbiol Biotechnol* 2007, 13:1–11.
2. Repoila F, Darfeuille F. Small regulatory non-coding RNAs in bacteria: physiology and mechanistic aspects. *Biol Cell* 2009, 101:117–131.
3. Shimoni Y, Friedlander G, Hetzroni G, Niv G, Altuvia S, Biham O, Margalit H. Regulation of gene expression by small non-coding RNAs: a quantitative view. *Mol Syst Biol* 2007, 3:138.
4. Molin C, Jauhiainen A, Warringer J, Nerman O, Sunnerhagen P. mRNA stability changes precede changes in steady-state mRNA amounts during hyperosmotic stress. *RNA* 2009, 15:600–614.
5. Wang Y, Liu CL, Storey JD, Tibshirani RJ, Herschlag D, Brown PO. Precision and functional specificity in mRNA decay. *Proc Natl Acad Sci U S A* 2002, 99:5860–5865.
6. García-Martínez J, Aranda A, Pérez-Ortín JE. Genomic run-on evaluates transcription rates for all yeast genes and identifies gene regulatory mechanisms. *Mol Cell* 2004, 15:303–313.
7. Grigull J, Mnaimneh S, Pootoolal J, Robinson MD, Hughes TR. Genome-wide analysis of mRNA stability using transcription inhibitors and microarrays reveals posttranscriptional control of ribosome biogenesis factors. *Mol Cell Biol* 2004, 24:5534–5547.
8. Bernstein JA, Khodursky AB, Lin PH, Lin-Chao S, Cohen SN. Global analysis of mRNA decay and abundance in *Escherichia coli* at single-gene resolution using two-color fluorescent DNA microarrays. *Proc Natl Acad Sci U S A* 2002, 99:9697–9702.
9. Selinger DW, Saxena RM, Cheung KJ, Church GM, Rosenow C. Global RNA half-life analysis in *Escherichia coli* reveals positional patterns of transcript degradation. *Genome Res* 2003, 13:216–223.
10. Hambraeus G, von Wachenfeldt C, Hederstedt L. Genome-wide survey of mRNA half-lives in *Bacillus subtilis* identifies extremely stable mRNAs. *Mol Genet Genomics* 2003, 269:706–714.
11. Redon E, Loubière P, Cocaign-Bousquet M. Role of mRNA stability during genome-wide adaptation of *Lactococcus lactis* to carbon starvation. *J Biol Chem* 2005, 280:36380–36385.
12. Anderson KL, Roberts C, Disz T, Vonstein V, Hwang K, Overbeek R, Olson PD, Projan SJ, Dunman PM. Characterization of the *Staphylococcus aureus* heat shock, cold shock, stringent, and SOS responses and their effects on log-phase mRNA turnover. *J Bacteriol* 2006, 188:6739–6756.
13. Janga SC, Babu MM. Transcript stability in the protein interaction network of *Escherichia coli*. *Mol Biosyst* 2009, 5:154–162.
14. Massé E, Escorcia FE, Gottesman S. Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. *Genes Dev* 2003, 17:2374–2383.
15. Viegas SC, Arraiano CM. Regulating the regulators: how ribonucleases dictate the rules in the control of small non-coding RNAs. *RNA Biol* 2008, 5:230–243.
16. Vogel J, Bartels V, Tang TH, Churakov G, Slagter-Jäger JG, Hüttenhofer A, Wagner EG. RNomics in *Escherichia coli* detects new sRNA species and indicates parallel transcriptional output in bacteria. *Nucleic Acids Res* 2003, 31:6435–6443.
17. Hutzinger R, Mrazek J, Vorwerk S, Huttenhofer A. NcRNA-microchip analysis: a novel approach to identify differential expression of non-coding RNAs. *RNA Biol* 2010, 7:81–90.
18. Arraiano CM, Andrade JM, Domingues S, Guinote IB, Malecki M, Matos RG, Moreira RN, Pobre V, Reis FP, Saramago M, et al. The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol Rev* 2010, 34:883–923.
19. Arraiano CM, Yancey SD, Kushner SR. Stabilization of discrete mRNA breakdown products in ams pnp rnb multiple mutants of *Escherichia coli* K-12. *J Bacteriol* 1988, 170:4625–4633.
20. Mackie GA. Secondary structure of the mRNA for ribosomal protein S20. Implications for cleavage by ribonuclease E. *J Biol Chem* 1992, 267:1054–1061.
21. McDowall KJ, Lin-Chao S, Cohen SN. A+U content rather than a particular nucleotide order determines the specificity of RNase E cleavage. *J Biol Chem* 1994, 269:10790–10796.
22. McDowall KJ, Hernandez RG, Lin-Chao S, Cohen SN. The *ams-1* and *rne-3071* temperature-sensitive mutations in the *ams* gene are in close proximity to each other and cause substitutions within a domain that resembles a product of the *Escherichia coli mre* locus. *J Bacteriol* 1993, 175:4245–4249.
23. Carpousis AJ, Luisi BF, McDowall KJ. Endonucleolytic initiation of mRNA decay in *Escherichia coli*. *Prog Mol Biol Transl Sci* 2009, 85:91–135.
24. Baker KE, Mackie GA. Ectopic RNase E sites promote bypass of 5′-end-dependent mRNA decay in *Escherichia coli*. *Mol Microbiol* 2003, 47:75–88.
25. Kime L, Jourdan SS, Stead JA, Hidalgo-Sastre A, McDowall KJ. Rapid cleavage of RNA by RNase E in the absence of 5′ monophosphate stimulation. *Mol Microbiol* 2010, 76:590–604.
26. Deana A, Celesnik H, Belasco JG. The bacterial enzyme RppH triggers messenger RNA degradation by 5′ pyrophosphate removal. *Nature* 2008, 451:355–358.
27. Kaberdin VR, Miczak A, Jakobsen JS, Lin-Chao S, McDowall KJ, von Gabain A. The endoribonucleolytic N-terminal half of *Escherichia coli* RNase E

is evolutionarily conserved in *Synechocystis* sp. and other bacteria but not the C-terminal half, which is sufficient for degradosome assembly. *Proc Natl Acad Sci U S A* 1998, 95:11637–11642.

28. Liou GG, Jane WN, Cohen SN, Lin NS, Lin-Chao S. RNA degradosomes exist in vivo in *Escherichia coli* as multicomponent complexes associated with the cytoplasmic membrane via the N-terminal region of ribonuclease E. *Proc Natl Acad Sci U S A* 2001, 98:63–68.
29. Prud'homme-Genereux A, Beran RK, Iost I, Ramey CS, Mackie GA, Simons RW. Physical and functional interactions among RNase E, polynucleotide phosphorylase and the cold-shock protein, CsdA: evidence for a 'cold shock degradosome'. *Mol Microbiol* 2004, 54:1409–1421.
30. Gao J, Lee K, Zhao M, Qiu J, Zhan X, Saxena A, Moore CJ, Cohen SN, Georgiou G. Differential modulation of *E. coli* mRNA abundance by inhibitory proteins that alter the composition of the degradosome. *Mol Microbiol* 2006, 61:394–406.
31. Py B, Higgins CF, Krisch HM, Carpousis AJ. A DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome. *Nature* 1996, 381:169–172.
32. Carpousis AJ, Van Houwe G, Ehretsmann C, Krisch HM. Copurification of *E. coli* RNAase E and PNPase: evidence for a specific association between two enzymes important in RNA processing and degradation. *Cell* 1994, 76:889–900.
33. Purusharth RI, Klein F, Sulthana S, Jager S, Jagannadham MV, Evguenieva-Hackenberg E, Ray MK, Klug G. Exoribonuclease R interacts with endoribonuclease E and an RNA helicase in the psychrotrophic bacterium *Pseudomonas syringae* Lz4W. *J Biol Chem* 2005, 280:14572–14578.
34. Andrade JM, Pobre V, Silva IJ, Domingues S, Arraiano CM. The role of 3′–5′ exoribonucleases in RNA degradation. *Prog Mol Biol Transl Sci* 2009, 85:187–229.
35. Stickney LM, Hankins JS, Miao X, Mackie GA. Function of the conserved S1 and KH domains in polynucleotide phosphorylase. *J Bacteriol* 2005, 187:7214–7221.
36. Spickler C, Mackie GA. Action of RNase II and polynucleotide phosphorylase against RNAs containing stem-loops of defined structure. *J Bacteriol* 2000, 182:2422–2427.
37. Liou GG, Chang HY, Lin CS, Lin-Chao S. DEAD box RhlB RNA helicase physically associates with exoribonuclease PNPase to degrade double-stranded RNA independent of the degradosome-assembling region of RNase E. *J Biol Chem* 2002, 277:41157–41162.
38. Falaleeva MV, Chetverina HV, Ugarov VI, Uzlova EA, Chetverin AB. Factors influencing RNA degradation by *Thermus thermophilus* polynucleotide phosphorylase. *FEBS J* 2008, 275:2214–2226.
39. Vincent HA, Deutscher MP. Substrate recognition and catalysis by the exoribonuclease RNase R. *J Biol Chem* 2006, 281:29769–29775.
40. Lisitsky I, Schuster G. Preferential degradation of polyadenylated and polyuridinylated RNAs by the bacterial exoribonuclease polynucleotide phosphorylase. *Eur J Biochem* 1999, 261:468–474.
41. Marujo PE, Hajnsdorf E, Le Derout J, Andrade R, Arraiano CM, Régnier P. RNase II removes the oligo(A) tails that destabilize the *rpsO* mRNA of *Escherichia coli*. *RNA* 2000, 6:1185–1193.
42. Cheng ZF, Deutscher MP. Quality control of ribosomal RNA mediated by polynucleotide phosphorylase and RNase R. *Proc Natl Acad Sci U S A* 2003, 100:6388–6393.
43. Mohanty BK, Kushner SR. Genomic analysis in *Escherichia coli* demonstrates differential roles for polynucleotide phosphorylase and RNase II in mRNA abundance and decay. *Mol Microbiol* 2003, 50:645–658.
44. Deutscher MP, Reuven NB. Enzymatic basis for hydrolytic versus phosphorolytic mRNA degradation in *Escherichia coli* and *Bacillus subtilis*. *Proc Natl Acad Sci U S A* 1991, 88:3277–3280.
45. Charpentier X, Faucher SP, Kalachikov S, Shuman HA. Loss of RNase R induces competence development in *Legionella pneumophila*. *J Bacteriol* 2008, 190:8126–8136.
46. Domingues S, Matos RG, Reis FP, Fialho AM, Barbas A, Arraiano CM. Biochemical characterization of the RNase II family of exoribonucleases from the human pathogens *Salmonella typhimurium* and *Streptococcus pneumoniae*. *Biochemistry* 2009, 48:11848–11857.
47. Bralley P, Gust B, Chang S, Chater KF, Jones GH. RNA 3′-tail synthesis in *Streptomyces*: *in vitro* and *in vivo* activities of RNase PH, the SCO3896 gene product and polynucleotide phosphorylase. *Microbiology* 2006, 152:627–636.
48. Hutchison CA, Peterson SN, Gill SR, Cline RT, White O, Fraser CM, Smith HO, Venter JC. Global transposon mutagenesis and a minimal *Mycoplasma* genome. *Science* 1999, 286:2165–2169.
49. Ghosh S, Deutscher MP. Oligoribonuclease is an essential component of the mRNA decay pathway. *Proc Natl Acad Sci U S A* 1999, 96:4372–4377.
50. Niyogi SK, Datta AK. A novel oligoribonuclease of *Escherichia coli*. I. Isolation and properties. *J Biol Chem* 1975, 250:7307–7312.
51. Fang M, Zeisberg WM, Condon C, Ogryzko V, Danchin A, Mechold U. Degradation of nanoRNA is performed by multiple redundant RNases in *Bacillus subtilis*. *Nucleic Acids Res* 2009, 37:5114–5125.
52. Jaskiewicz L, Filipowicz W. Role of Dicer in posttranscriptional RNA silencing. *Curr Top Microbiol Immunol* 2008, 320:77–97.

53. Babitzke P, Granger L, Olszewski J, Kushner SR. Analysis of mRNA decay and rRNA processing in *Escherichia coli* multiple mutants carrying a deletion in RNase III. *J Bacteriol* 1993, 175:229–239.
54. Herskovitz MA, Bechhofer DH. Endoribonuclease RNase III is essential in *Bacillus subtilis*. *Mol Microbiol* 2000, 38:1027–1033.
55. Redko Y, Bechhofer DH, Condon C. Mini-III, an unusual member of the RNase III family of enzymes, catalyses 23S ribosomal RNA maturation in *B. subtilis*. *Mol Microbiol* 2008, 68:1096–1106.
56. Bechhofer DH. Messenger RNA decay and maturation in *Bacillus subtilis*. *Prog Mol Biol Transl Sci* 2009, 85:231–273.
57. Viegas SC, Silva IJ, Saramago M, Domingues S, Arraiano CM. Regulation of the small regulatory RNA MicA by ribonuclease III: a target-dependent pathway. *Nucleic Acids Res* 2010, 39:2918–2930.
58. Even S, Pellegrini O, Zig L, Labas V, Vinh J, Bréchemmier-Baey D, Putzer H. Ribonucleases J1 and J2: two novel endoribonucleases in *B. subtilis* with functional homology to *E. coli* RNase E. *Nucleic Acids Res* 2005, 33:2141–2152.
59. Madhugiri R, Evguenieva-Hackenberg E. RNase J is involved in the 5′-end maturation of 16S rRNA and 23S rRNA in *Sinorhizobium meliloti*. *FEBS Lett* 2009, 583:2339–2342.
60. de la Sierra-Gallay IL, Zig L, Jamalli A, Putzer H. Structural insights into the dual activity of RNase J. *Nat Struct Mol Biol* 2008, 15:206–212.
61. Mäder U, Zig L, Kretschmer J, Homuth G, Putzer H. mRNA processing by RNases J1 and J2 affects *Bacillus subtilis* gene expression on a global scale. *Mol Microbiol* 2008, 70:183–196.
62. Mathy N, Bénard L, Pellegrini O, Daou R, Wen T, Condon C. 5′-to-3′ exoribonuclease activity in bacteria: role of RNase J1 in rRNA maturation and 5′ stability of mRNA. *Cell* 2007, 129:681–692.
63. Mathy N, Hébert A, Mervelet P, Bénard L, Dorléans A, de la Sierra-Gallay IL, Noirot P, Putzer H, Condon C. *Bacillus subtilis* ribonucleases J1 and J2 form a complex with altered enzyme behaviour. *Mol Microbiol* 2010, 75:489–498.
64. Shahbabian K, Jamalli A, Zig L, Putzer H. RNase Y, a novel endoribonuclease, initiates riboswitch turnover in *Bacillus subtilis*. *Embo J* 2009, 28:3523–3533.
65. Commichau FM, Rothe FM, Herzberg C, Wagner E, Hellwig D, Lehnik-Habrink M, Hammer E, Völker U, Stülke J. Novel activities of glycolytic enzymes in *Bacillus subtilis*: interactions with essential proteins involved in mRNA processing. *Mol Cell Proteomics* 2009, 8:1350–1360.
66. Richards J, Sundermeier T, Svetlanov A, Karzai AW. Quality control of bacterial mRNA decoding and decay. *Biochim Biophys Acta* 2008, 1779:574–582.
67. Keiler KC. Biology of trans-translation. *Annu Rev Microbiol* 2008, 62:133–151.
68. Garza-Sánchez F, Shoji S, Fredrick K, Hayes CS. RNase II is important for A-site mRNA cleavage during ribosome pausing. *Mol Microbiol* 2009, 73:882–897.
69. Karzai AW, Sauer RT. Protein factors associated with the SsrA.SmpB tagging and ribosome rescue complex. *Proc Natl Acad Sci U S A* 2001, 98:3040–3044.
70. Cairrão F, Cruz A, Mori H, Arraiano CM. Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. *Mol Microbiol* 2003, 50:1349–1360.
71. Ge Z, Mehta P, Richards J, Karzai AW. Non-stop mRNA decay initiates at the ribosome. *Mol Microbiol* 2010, 78:1159–1170.
72. Liang W, Deutscher MP. A novel mechanism for ribonuclease regulation: transfer-messenger RNA (tmRNA) and its associated protein SmpB regulate the stability of RNase R. *J Biol Chem* 2010, 285:29054–29058.
73. Li Z, Reimers S, Pandit S, Deutscher MP. RNA quality control: degradation of defective transfer RNA. *EMBO J* 2002, 21:1132–1138.
74. Callaghan AJ, Marcaida MJ, Stead JA, McDowall KJ, Scott WG, Luisi BF. Structure of *Escherichia coli* RNase E catalytic domain and implications for RNA turnover. *Nature* 2005, 437:1187–1191.
75. Koslover DJ, Callaghan AJ, Marcaida MJ, Garman EF, Martick M, Scott WG, Luisi BF. The crystal structure of the *Escherichia coli* RNase E apoprotein and a mechanism for RNA degradation. *Structure* 2008, 16:1238–1244.
76. Khemici V, Poljak L, Luisi BF, Carpousis AJ. The RNase E of *Escherichia coli* is a membrane-binding protein. *Mol Microbiol* 2008, 70:799–813.
77. Gan J, Tropea JE, Austin BP, Court DL, Waugh DS, Ji X. Structural insight into the mechanism of double-stranded RNA processing by ribonuclease III. *Cell* 2006, 124:355–366.
78. Pertzev AV, Nicholson AW. Characterization of RNA sequence determinants and antideterminants of processing reactivity for a minimal substrate of *Escherichia coli* ribonuclease III. *Nucleic Acids Res* 2006, 34:3708–3721.
79. Gan J, Shaw G, Tropea JE, Waugh DS, Court DL, Ji X. A stepwise model for double-stranded RNA processing by ribonuclease III. *Mol Microbiol* 2008, 67:143–154.
80. Blaszczyk J, Gan J, Tropea JE, Court DL, Waugh DS, Ji X. Noncatalytic assembly of ribonuclease III with double-stranded RNA. *Structure* 2004, 12:457–466.
81. Lebreton A, Tomecki R, Dziembowski A, Séraphin B. Endonucleolytic RNA cleavage by a eukaryotic exosome. *Nature* 2008, 456:993–996.

82. Amblar M, Barbas A, Fialho AM, Arraiano CM. Characterization of the functional domains of *Escherichia coli* RNase II. *J Mol Biol* 2006, 360:921–933.

83. Frazão C, McVey CE, Amblar M, Barbas A, Vonrhein C, Arraiano CM, Carrondo MA. Unravelling the dynamics of RNA degradation by ribonuclease II and its RNA-bound complex. *Nature* 2006, 443:110–114.

84. Zuo Y, Vincent HA, Zhang J, Wang Y, Deutscher MP, Malhotra A. Structural basis for processivity and single-strand specificity of RNase II. *Mol Cell* 2006, 24:149–156.

85. Matos RG, Barbas A, Arraiano CM. RNase R mutants elucidate the catalysis of structured RNA: RNA-binding domains select the RNAs targeted for degradation. *Biochem J* 2009, 423:291–301.

86. Vincent HA, Deutscher MP. The roles of individual domains of RNase R in substrate binding and exoribonuclease activity. The nuclease domain is sufficient for digestion of structured RNA. *J Biol Chem* 2009, 284:486–494.

87. Barbas A, Matos RG, Amblar M, López-Viñas E, Gomez-Puertas P, Arraiano CM. New insights into the mechanism of RNA degradation by ribonuclease II: identification of the residue responsible for setting the RNase II end product. *J Biol Chem* 2008, 283:13070–13076.

88. Amblar M, Arraiano CM. A single mutation in *Escherichia coli* ribonuclease II inactivates the enzyme without affecting RNA binding. *FEBS J* 2005, 272:363–374.

89. Barbas A, Matos RG, Amblar M, López-Viñas E, Gomez-Puertas P, Arraiano CM. Determination of key residues for catalysis and RNA cleavage specificity: one mutation turns RNase II into a "SUPER-ENZYME". *J Biol Chem* 2009, 284:20486–20498.

90. Awano N, Rajagopal V, Arbing M, Patel S, Hunt J, Inouye M, Phadtare S. *Escherichia coli* RNase R has dual activities, helicase and RNase. *J Bacteriol* 2010, 192:1344–1352.

91. Vincent HA, Deutscher MP. Insights into how RNase R degrades structured RNA: analysis of the nuclease domain. *J Mol Biol* 2009, 387:570–583.

92. Pruijn GJ. Doughnuts dealing with RNA. *Nat Struct Mol Biol* 2005, 12:562–564.

93. Symmons MF, Jones GH, Luisi BF. A duplicated fold is the structural basis for polynucleotide phosphorylase catalytic activity, processivity, and regulation. *Structure* 2000, 8:1215–1226.

94. Nurmohamed S, Vaidialingam B, Callaghan AJ, Luisi BF. Crystal structure of *Escherichia coli* polynucleotide phosphorylase core bound to RNase E, RNA and manganese: implications for catalytic mechanism and RNA degradosome assembly. *J Mol Biol* 2009, 389:17–33.

95. Shi Z, Yang WZ, Lin-Chao S, Chak KF, Yuan HS. Crystal structure of *Escherichia coli* PNPase: central channel residues are involved in processive RNA degradation. *RNA* 2008, 14:2361–2371.

96. Jarrige A, Bréchemier-Baey D, Mathy N, Duché O, Portier C. Mutational analysis of polynucleotide phosphorylase from *Escherichia coli*. *J Mol Biol* 2002, 321:397–409.

97. Briani F, Del Favero M, Capizzuto R, Consonni C, Zangrossi S, Greco C, De Gioia L, Tortora P, Dehò G. Genetic analysis of polynucleotide phosphorylase structure and functions. *Biochimie* 2007, 89:145–157.

98. Matus-Ortega ME, Regonesi ME, Piña-Escobedo A, Tortora P, Dehò G, García-Mena J. The KH and S1 domains of *Escherichia coli* polynucleotide phosphorylase are necessary for autoregulation and growth at low temperature. *Biochim Biophys Acta* 2007, 1769:194–203.

99. Amblar M, Barbas A, Gomez-Puertas P, Arraiano CM. The role of the S1 domain in exoribonucleolytic activity: substrate specificity and multimerization. *RNA* 2007, 13:317–327.

100. Nurmohamed S, Vincent HA, Titman CM, Chandran V, Pears MR, Du D, Griffin JL, Callaghan AJ, Luisi BF. Polynucleotide phosphorylase activity may be modulated by metabolites in *Escherichia coli*. *J Biol Chem* 2011, 286:14315–14323.

101. Houseley J, Tollervey D. The many pathways of RNA degradation. *Cell* 2009, 136:763–776.

102. Mohanty BK, Kushner SR. The majority of *Escherichia coli* mRNAs undergo post-transcriptional modification in exponentially growing cells. *Nucleic Acids Res* 2006, 34:5695–5704.

103. Dreyfus M, Régnier P. The poly(A) tail of mRNAs: bodyguard in eukaryotes, scavenger in bacteria. *Cell* 2002, 111:611–613.

104. August JT, Ortiz PJ, Hurwitz J. Ribonucleic acid-dependent ribonucleotide incorporation. I. Purification and properties of the enzyme. *J Biol Chem* 1962, 237:3786–3793.

105. Mohanty BK, Kushner SR. Bacterial/archaeal/organellar polyadenylation. *Wiley Interdiscip Rev: RNA* 2011, 2:256–276.

106. Mohanty BK, Maples VF, Kushner SR. The Sm-like protein Hfq regulates polyadenylation dependent mRNA decay in *Escherichia coli*. *Mol Microbiol* 2004, 54:905–920.

107. O'Hara EB, Chekanova JA, Ingle CA, Kushner ZR, Peters E, Kushner SR. Polyadenylation helps regulate mRNA decay in *Escherichia coli*. *Proc Natl Acad Sci U S A* 1995, 92:1807–1811.

108. Cao GJ, Sarkar N. Identification of the gene for an *Escherichia coli* poly(A) polymerase. *Proc Natl Acad Sci U S A* 1992, 89:10380–10384.

109. Mohanty BK, Kushner SR. Polynucleotide phosphorylase functions both as a 3′ right-arrow 5′ exonuclease

and a poly(A) polymerase in *Escherichia coli*. *Proc Natl Acad Sci U S A* 2000, 97:11966–11971.

110. Tuckerman JR, Gonzalez G, Gilles-Gonzalez MA. Cyclic di-GMP activation of polynucleotide phosphorylase signal-dependent RNA processing. *J Mol Biol* 2011, 407:633–639.
111. Slomovic S, Schuster G. Exonucleases and endonucleases involved in polyadenylation-assisted RNA decay. *Wiley Interdiscip Rev: RNA* 2011, 2:106–123.
112. Gopalakrishna Y, Langley D, Sarkar N. Detection of high levels of polyadenylate-containing RNA in bacteria by the use of a single-step RNA isolation procedure. *Nucleic Acids Res* 1981, 9:3545–3554.
113. Campos-Guillén J, Bralley P, Jones GH, Bechhofer DH, Olmedo-Alvarez G. Addition of poly(A) and heteropolymeric 3′ ends in *Bacillus subtilis* wild-type and polynucleotide phosphorylase-deficient strains. *J Bacteriol* 2005, 187:4698–4706.
114. Cao GJ, Sarkar N. Stationary phase-specific mRNAs in *Escherichia coli* are polyadenylated. *Biochem Biophys Res Commun* 1997, 239:46–50.
115. Goodrich AF, Steege DA. Roles of polyadenylation and nucleolytic cleavage in the filamentous phage mRNA processing and decay pathways in *Escherichia coli*. *RNA* 1999, 5:972–985.
116. Haugel-Nielsen J, Hajnsdorf E, Régnier P. The *rpsO* mRNA of *Escherichia coli* is polyadenylated at multiple sites resulting from endonucleolytic processing and exonucleolytic degradation. *EMBO J* 1996, 15:3144–3152.
117. Lupold DS, Caoile AG, Stern DB. Polyadenylation occurs at multiple sites in maize mitochondrial *cox2* mRNA and is independent of editing status. *Plant Cell* 1999, 11:1565–1578.
118. Perrin R, Lange H, Grienenberger JM, Gagliardi D. AtmtPNPase is required for multiple aspects of the 18S rRNA metabolism in *Arabidopsis thaliana* mitochondria. *Nucleic Acids Res* 2004, 32:5174–5182.
119. Feng Y, Cohen SN. Unpaired terminal nucleotides and 5′ monophosphorylation govern 3′ polyadenylation by *Escherichia coli* poly(A) polymerase I. *Proc Natl Acad Sci U S A* 2000, 97:6415–6420.
120. Mohanty BK, Kushner SR. Analysis of the function of *Escherichia coli* poly(A) polymerase I in RNA metabolism. *Mol Microbiol* 1999, 34:1094–1108.
121. Xu F, Lin-Chao S, Cohen SN. The *Escherichia coli pcnB* gene promotes adenylylation of antisense RNAI of ColE1-type plasmids *in vivo* and degradation of RNAI decay intermediates. *Proc Natl Acad Sci U S A* 1993, 90:6756–6760.
122. Argaman L, Hershberg R, Vogel J, Bejerano G, Wagner EG, Margalit H, Altuvia S. Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli*. *Curr Biol* 2001, 11:941–950.
123. Söderbom F, Binnie U, Masters M, Wagner EG. Regulation of plasmid R1 replication: PcnB and RNase E expedite the decay of the antisense RNA, CopA. *Mol Microbiol* 1997, 26:493–504.
124. Reichenbach B, Maes A, Kalamorz F, Hajnsdorf E, Görke B. The small RNA GlmY acts upstream of the sRNA GlmZ in the activation of *glmS* expression and is subject to regulation by polyadenylation in *Escherichia coli*. *Nucleic Acids Res* 2008, 36:2570–2580.
125. Viegas SC, Pfeiffer V, Sittka A, Silva IJ, Vogel J, Arraiano CM. Characterization of the role of ribonucleases in *Salmonella* small RNA decay. *Nucleic Acids Res* 2007, 35:7651–7664.
126. Dam Mikkelsen N, Gerdes K. Sok antisense RNA from plasmid R1 is functionally inactivated by RNase E and polyadenylated by poly(A) polymerase I. *Mol Microbiol* 1997, 26:311–320.
127. Raynal LC, Carpousis AJ. Poly(A) polymerase I of *Escherichia coli*: characterization of the catalytic domain, an RNA binding site and regions for the interaction with proteins involved in mRNA degradation. *Mol Microbiol* 1999, 32:765–775.
128. Carabetta VJ, Mohanty BK, Kushner SR, Silhavy TJ. The response regulator SprE (RssB) modulates polyadenylation and mRNA stability in *Escherichia coli*. *J Bacteriol* 2009, 191:6812–6821.
129. Jasiecki J, Wegrzyn G. Localization of *Escherichia coli* poly(A) polymerase I in cellular membrane. *Biochem Biophys Res Commun* 2005, 329:598–602.
130. Carabetta VJ, Silhavy TJ, Cristea IM. The response regulator SprE (RssB) is required for maintaining poly(A) polymerase I-degradosome association during stationary phase. *J Bacteriol* 2010, 192:3713–3721.
131. Kajitani M, Kato A, Wada A, Inokuchi Y, Ishihama A. Regulation of the *Escherichia coli* hfq gene encoding the host factor for phage Q beta. *J Bacteriol* 1994, 176:531–534.
132. Mikulecky PJ, Kaw MK, Brescia CC, Takach JC, Sledjeski DD, Feig AL. *Escherichia coli* Hfq has distinct interaction surfaces for DsrA, *rpoS* and poly(A) RNAs. *Nat Struct Mol Biol* 2004, 11:1206–1214.
133. Hajnsdorf E, Régnier P. Host factor Hfq of *Escherichia coli* stimulates elongation of poly(A) tails by poly(A) polymerase I. *Proc Natl Acad Sci U S A* 2000, 97:1501–1505.
134. Le Derout J, Folichon M, Briani F, Dehò G, Régnier P, Hajnsdorf E. Hfq affects the length and the frequency of short oligo(A) tails at the 3′ end of *Escherichia coli rpsO* mRNAs. *Nucleic Acids Res* 2003, 31:4017–4023.
135. Folichon M, Arluison V, Pellegrini O, Huntzinger E, Régnier P, Hajnsdorf E. The poly(A) binding protein Hfq protects RNA from RNase E and exoribonucleolytic degradation. *Nucleic Acids Res* 2003, 31:7302–7310.

136. Morita T, Maki K, Aiba H. RNase E-based ribonucleoprotein complexes: mechanical basis of mRNA destabilization mediated by bacterial noncoding RNAs. *Genes Dev* 2005, 19:2176–2186.

137. Tsui HC, Leung HC, Winkler ME. Characterization of broadly pleiotropic phenotypes caused by an hfq insertion mutation in *Escherichia coli* K-12. *Mol Microbiol* 1994, 13:35–49.

138. Johansen J, Rasmussen AA, Overgaard M, Valentin-Hansen P. Conserved small non-coding RNAs that belong to the sigmaE regulon: role in down-regulation of outer membrane proteins. *J Mol Biol* 2006, 364:1–8.

139. Waters LS, Storz G. Regulatory RNAs in bacteria. *Cell* 2009, 136:615–628.

140. Geissmann TA, Touati D. Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. *EMBO J* 2004, 23:396–405.

141. Moll I, Leitsch D, Steinhauser T, Bläsi U. RNA chaperone activity of the Sm-like Hfq protein. *EMBO Rep* 2003, 4:284–289.

142. Link TM, Valentin-Hansen P, Brennan RG. Structure of *Escherichia coli* Hfq bound to polyriboadenylate RNA. *Proc Natl Acad Sci U S A* 2009, 106: 19292–19297.

143. Babitzke P, Romeo T. CsrB sRNA family: sequestration of RNA-binding regulatory proteins. *Curr Opin Microbiol* 2007, 10:156–163.

144. Massé E, Majdalani N, Gottesman S. Regulatory roles for small RNAs in bacteria. *Curr Opin Microbiol* 2003, 6:120–124.

145. Darfeuille F, Unoson C, Vogel J, Wagner EG. An antisense RNA inhibits translation by competing with standby ribosomes. *Mol Cell* 2007, 26:381–392.

146. Sharma CM, Darfeuille F, Plantinga TH, Vogel J. A small RNA regulates multiple ABC transporter mRNAs by targeting C/A-rich elements inside and upstream of ribosome-binding sites. *Genes Dev* 2007, 21:2804–2817.

147. Vecerek B, Moll I, Bläsi U. Control of Fur synthesis by the non-coding RNA RyhB and iron-responsive decoding. *EMBO J* 2007, 26:965–975.

148. Pfeiffer V, Papenfort K, Lucchini S, Hinton JC, Vogel J. Coding sequence targeting by MicC RNA reveals bacterial mRNA silencing downstream of translational initiation. *Nat Struct Mol Biol* 2009, 16:840–846.

149. Majdalani N, Cunning C, Sledjeski D, Elliott T, Gottesman S. DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription. *Proc Natl Acad Sci U S A* 1998, 95:12462–12467.

150. Majdalani N, Hernandez D, Gottesman S. Regulation and mode of action of the second small RNA activator of RpoS translation, RprA. *Mol Microbiol* 2002, 46:813–826.

151. Soper T, Mandin P, Majdalani N, Gottesman S, Woodson SA. Positive regulation by small RNAs and the role of Hfq. *Proc Natl Acad Sci U S A* 2010, 107:9602–9607.

152. Udekwu KI. Transcriptional and post-transcriptional regulation of the *Escherichia coli luxS* mRNA; involvement of the sRNA MicA. *PLoS One* 2010, 5:e13449.

153. Brantl S. Regulatory mechanisms employed by *cis*-encoded antisense RNAs. *Curr Opin Microbiol* 2007, 10:102–109.

154. Rasmussen AA, Johansen J, Nielsen JS, Overgaard M, Kallipolitis B, Valentin-Hansen P. A conserved small RNA promotes silencing of the outer membrane protein YbfM. *Mol Microbiol* 2009, 72:566–577.

155. Nielsen JS, Lei LK, Ebersbach T, Olsen AS, Klitgaard JK, Valentin-Hansen P, Kallipolitis BH. Defining a role for Hfq in Gram-positive bacteria: evidence for Hfq-dependent antisense regulation in *Listeria monocytogenes*. *Nucleic Acids Res* 2010, 38: 907–919.

156. Jain C, Belasco JG. RNase E autoregulates its synthesis by controlling the degradation rate of its own mRNA in *Escherichia coli*: unusual sensitivity of the *rne* transcript to RNase E activity. *Genes Dev* 1995, 9:84–96.

157. Bardwell JC, Régnier P, Chen SM, Nakamura Y, Grunberg-Manago M, Court DL. Autoregulation of RNase III operon by mRNA processing. *EMBO J* 1989, 8:3401–3407.

158. Portier C, Dondon L, Grunberg-Manago M, Régnier P. The first step in the functional inactivation of the *Escherichia coli* polynucleotide phosphorylase messenger is a ribonuclease III processing at the 5′ end. *EMBO J* 1987, 6:2165–2170.

159. Carzaniga T, Briani F, Zangrossi S, Merlino G, Marchi P, Dehò G. Autogenous regulation of *Escherichia coli* polynucleotide phosphorylase expression revisited. *J Bacteriol* 2009, 191:1738–1748.

160. Zilhão R, Plumbridge J, Hajnsdorf E, Régnier P, Arraiano CM. *Escherichia coli* RNase II: characterization of the promoters involved in the transcription of *rnb*. *Microbiology* 1996, 142(Pt 2):367–375.

161. Zilhão R, Cairrão F, Régnier P, Arraiano CM. PNPase modulates RNase II expression in *Escherichia coli*: implications for mRNA decay and cell metabolism. *Mol Microbiol* 1996, 20:1033–1042.

162. Zilhão R, Régnier P, Arraiano CM. The role of endonucleases in the expression of ribonuclease II in *Escherichia coli*. *FEMS Microbiol Lett* 1995, 130:237–244.

163. Cairrão F, Arraiano CM. The role of endoribonucleases in the regulation of RNase R. *Biochem Biophys Res Commun* 2006, 343:731–737.

164. Lee K, Zhan X, Gao J, Qiu J, Feng Y, Meganathan R, Cohen SN, Georgiou G. RraA. A protein inhibitor of

RNase E activity that globally modulates RNA abundance in *E. coli*. *Cell* 2003, 114:623–634.

165. Le Derout J, Régnier P, Hajnsdorf E. Both temperature and medium composition regulate RNase E processing efficiency of the *rpsO* mRNA coding for ribosomal protein S15 of *Escherichia coli*. *J Mol Biol* 2002, 319:341–349.
166. Barlow T, Berkmen M, Georgellis D, Bayr L, Arvidson S, von Gabain A. RNase E, the major player in mRNA degradation, is down-regulated in *Escherichia coli* during a transient growth retardation (diauxic lag). *Biol Chem* 1998, 379:33–38.
167. Georgellis D, Arvidson S, von Gabain A. Decay of *ompA* mRNA and processing of 9S RNA are immediately affected by shifts in growth rate, but in opposite manners. *J Bacteriol* 1992, 174:5382–5390.
168. Chen C, Deutscher MP. Elevation of RNase R in response to multiple stress conditions. *J Biol Chem* 2005, 280:34393–34396.
169. Andrade JM, Cairrão F, Arraiano CM. RNase R affects gene expression in stationary phase: regulation of *ompA*. *Mol Microbiol* 2006, 60:219–228.
170. Piazza F, Zappone M, Sana M, Briani F, Dehò G. Polynucleotide phosphorylase of *Escherichia coli* is required for the establishment of bacteriophage P4 immunity. *J Bacteriol* 1996, 178:5513–5521.
171. Zangrossi S, Briani F, Ghisotti D, Regonesi ME, Tortora P, Dehò G. Transcriptional and post-transcriptional control of polynucleotide phosphorylase during cold acclimation in *Escherichia coli*. *Mol Microbiol* 2000, 36:1470–1480.
172. García-Mena J, Das A, Sánchez-Trujillo A, Portier C, Montañez C. A novel mutation in the KH domain of polynucleotide phosphorylase affects autoregulation and mRNA decay in *Escherichia coli*. *Mol Microbiol* 1999, 33:235–248.
173. Cairrão F, Chora A, Zilhão R, Carpousis AJ, Arraiano CM. RNase II levels change according to the growth conditions: characterization of *gmr*, a new *Escherichia coli* gene involved in the modulation of RNase II. *Mol Microbiol* 2001, 39:1550–1561.
174. Zilhão R, Caillet J, Régnier P, Arraiano CM. Precise physical mapping of the *Escherichia coli rnb* gene, encoding ribonuclease II. *Mol Gen Genet* 1995, 248:242–246.
175. Zilhão R, Camelo L, Arraiano CM. DNA sequencing and expression of the gene *rnb* encoding *Escherichia coli* ribonuclease II. *Mol Microbiol* 1993, 8:43–51.
176. Mohanty BK, Kushner SR. Polyadenylation of *Escherichia coli* transcripts plays an integral role in regulating intracellular levels of polynucleotide phosphorylase and RNase E. *Mol Microbiol* 2002, 45:1315–1324.
177. Nevo-Dinur K, Nussbaum-Shochat A, Ben-Yehuda S, Amster-Choder O. Translation-independent localization of mRNA in *E. coli*. *Science* 2011, 331:1081–1084.
178. Montero Llopis P, Jackson AF, Sliusarenko O, Surovtsev I, Heinritz J, Emonet T, Jacobs-Wagner C. Spatial organization of the flow of genetic information in bacteria. *Nature* 2010, 466:77–81.

FURTHER READING

Andrade JM, Pobre V, Silva IJ, Domingues S, Arraiano CM. The role of 3′-5′ exoribonucleases in RNA degradation. *Prog Mol Biol Transl Sci* 2009, 85:187–229.

Arraiano CM, Andrade JM, Domingues S, Guinote IB, Malecki M, et al. The critical role of RNA processing and degradation in the control of gene expression. *FEMS Microbiol Rev* 2010, 34:883–923.

Brantl S. Regulatory mechanisms employed by cis-encoded antisense RNAs. *Curr Opin Microbiol* 2007, 10:102–109.

Carpousis AJ, Luisi BF, McDowall KJ. Endonucleolytic initiation of mRNA decay in Escherichia coli. *Prog Mol Biol Transl Sci* 2009, 85:91–135.

Gottesman S, Storz G. Bacterial Small RNA Regulators: Versatile Roles and Rapidly Evolving Variations. *Cold Spring Harb Perspect Biol* 2010.

Ibrahim H, Wilusz J, Wilusz CJ. RNA recognition by 3′-to-5′ exonucleases: the substrate perspective. *Biochim Biophys Acta* 2008, 1779:256–265.

Matos RG, Pobre V, Reis PF, Malecki M, Andrade JM, Arraiano CM. Structure and degradation mechanisms of 3′ to 5′ exoribonucleases. In: Nicholson AW, ed., *Ribonucleases, Nucleic Acids and Molecular Biology*, Vol 26. Berlin, Heidelberg: Springer-Verlag; 2011.

Vogel J. A rough guide to the non-coding RNA world of Salmonella. *Mol Microbiol* 2009, 71:1–11.

Waters LS, Storz G. Regulatory RNAs in bacteria. *Cell* 2009, 136:615–628.

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